

Techniques in Life Science and Biomedicine for the Non-Expert
Series Editor: Alexander E. Kalyuzhny

Christine Goetz
Christopher Hammerbeck
Jody Bonnevier

Flow Cytometry Basics for the Non-Expert

EXTRAS ONLINE



Springer

Techniques in Life Science and Biomedicine for the Non-Expert

Series editor

Alexander E. Kalyuzhny, Bio-Techne, Inc., Minneapolis, MN, USA

The goal of this series is to provide concise but thorough introductory guides to various scientific techniques, aimed at both the non-expert researcher and novice scientist. Each book will highlight the advantages and limitations of the technique being covered, identify the experiments to which the technique is best suited, and include numerous figures to help better illustrate and explain the technique to the reader. Currently, there is an abundance of books and journals offering various scientific techniques to experts, but these resources, written in technical scientific jargon, can be difficult for the non-expert, whether an experienced scientist from a different discipline or a new researcher, to understand and follow. These techniques, however, may in fact be quite useful to the non-expert due to the interdisciplinary nature of numerous disciplines, and the lack of sufficient comprehensible guides to such techniques can and does slow down research and lead to employing inadequate techniques, resulting in inaccurate data. This series sets out to fill the gap in this much needed scientific resource.

More information about this series at <http://www.springer.com/series/13601>

Christine Goetz • Christopher Hammerbeck
Jody Bonnevier

Flow Cytometry Basics for the Non-Expert

 Springer

Christine Goetz
Department of Antibody Development
R&D Systems/Bio-Techne
Minneapolis, MN, USA

Christopher Hammerbeck
Department of Antibody Development
R&D Systems/Bio-Techne
Minneapolis, MN, USA

Jody Bonnevier
Department of Antibody Development
R&D Systems/Bio-Techne
Minneapolis, MN, USA

ISSN 2367-1114 ISSN 2367-1122 (electronic)
Techniques in Life Science and Biomedicine for the Non-Expert
ISBN 978-3-319-98070-6 ISBN 978-3-319-98071-3 (eBook)
<https://doi.org/10.1007/978-3-319-98071-3>

Library of Congress Control Number: 2018953158

© Springer Nature Switzerland AG 2018

This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.

The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, express or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Anyone who has spent a significant amount of time in an immunology lab knows the contributions that flow cytometry has brought to that field. But, before any one of those discoveries could be made, the scientists performing those experiments needed to understand the intricacies of flow cytometry. Like any scientific technique, it can take time to learn and master the process. And no matter how proficient one becomes, everyone has a story about their first experience performing a flow cytometry experiment. In the spirit of full disclosure, of the three of us, only one of us actually had a successful first flow experiment. In fact, it took a good 6 experiments before the other two of us had anything resembling a successful flow cytometry experiment. Among some of the more “important” flow cytometry lessons we’ve learned over time include (1) cells and bleach don’t play well together, (2) sheath line filters do not withstand overly stressed graduate students, (3) if your cells look like cottage cheese, do not run them through the flow cytometer, (4) remember your alphabet when fixing and permeabilizing your cells—F comes before P or you’re starting over, and (5) cleanliness is next to godliness but don’t flame sterilize the whole tissue culture hood when culturing cells (true story). Our mentors had the patience of saints.

When we first joined the MICaB program at the University of Minnesota in the mid- to late-1990s and early 2000s, cutting edge was a one laser, 3-color BD FACScan™ and a two laser, 4-color BD FACSCalibur™. There were no formal flow cytometry training courses. At that time, the description of flow cytometry in textbooks was rudimentary at best and there were no “how-to” books about flow either. If you were more adept at learning visually you were out of luck because the early descriptions of flow cytometry in textbooks were worthless; there were no visual examples of data plots.

You learned from others and fortunately for us, we had some very good mentors. They taught us the right way to use a flow cytometer and the right way to perform a flow experiment, but somehow, they never shied away from reminding everyone that we clogged the cytometer or showing us how our uncompensated data looked more like a Picasso painting than a flow cytometry plot. Ah, the good old days. Of course, mentoring has its disadvantages. One, it takes a lot of time for both the

master and apprentice, and let's be honest, if you're just learning something, you don't know what you don't know, and it's hard to ask questions about the things you don't know. And two, inevitably, each mentor usually has a slightly different way of doing things. Individuality is the enemy of standardization and can often introduce errors, misconceptions, and poor technique.

Things have improved. Most major research institutes now offer standardized flow cytometry courses before using cytometers. Many offer flow cytometry cores so you can easily get help or have them run your samples for you. (Back in the day, you were your own flow core). Still, not everyone that wants to learn about flow cytometry attends a major research university. Not everyone is going to have access to a mentor that knows how to perform a flow cytometry experiment correctly and not everyone who knows how to correctly perform flow cytometry has the time, ability, or desire to teach an apprentice. Not everyone needs to know how to perform a correct flow experiment but still needs to know how the process works and how to correctly interpret a flow cytometry experiment.

For those of you who are new to flow and fit these descriptions, this book was written with you in mind. In reflecting on our first encounters with flow cytometry, we had three simple goals: (1) present the information from a real user's perspective, (2) keep it basic but accurate, and (3) visually demonstrate the concepts. You have to start somewhere and, as we all know, the beginning is the best place to start. Rather than just summarize every bit of flow cytometry information that we could find, we've tried to tailor the breadth and depth of the topics herein to ones that we routinely feel are necessary to learn and teach how to perform flow cytometry correctly. Those topics that are vitally important to every flow cytometry experiment are discussed in more detail (we considered calling Chapters 5 and 7 "The Never-Ending Story") while other topics have not received as much attention. This means that there are more advanced topics that we haven't covered in this book and there are topics that we haven't covered in excruciating detail. It's not that these other areas are unimportant, but we've attempted to keep this book accessible to first-time flow cytometry users from as many backgrounds as possible. For those of you who are visual learners or aren't actually performing flow cytometry experiments, we've attempted to illustrate as many of the concepts as possible. It's easy to find examples of flow cytometry done correctly, but it's often just as important to know what it looks like when flow cytometry is not done correctly. We've purposely written the book in a more conversational tone, the way a mentor would interact with a mentee. Of course, this tone gets lost here and there (but even mentors have a rough day every now and then).

The concepts we discuss throughout the pages of this book are independent of any particular antibody vendor. That's not to say that products from one vendor may not give you slightly different results than products from a second vendor. To make this book and the data examples as user friendly as possible we've provided a list of the materials we've used in each chapter and included a chapter that specifically outlines the protocols that we used.

Success will not come overnight so start small. Start with one or two colors. Use the examples that we've outlined in this book and see if you can obtain similar

results. Feel free to experiment with the protocols to figure out what works best and what doesn't work at all. Try various fluorochromes combinations. Experiment with manually adjusting compensation values and see how that affects the positions of your cell populations. Don't be afraid to screw up, but when you do, try to understand what happened. There are many fantastic print and Internet resources that you can use to further your knowledge of flow cytometry should that be something you desire to pursue. Most of all, realize that there will be days when nothing will work no matter how well you designed and conducted the experiment. This usually ends up being the last experiment for your paper or thesis. That is why graduate school lasts 5–6 years for immunologists and only 2 years for chemical engineers. Good luck as you begin taking your first practical steps to beginning flow cytometry!

Minneapolis, MN, USA
Minneapolis, MN, USA
Minneapolis, MN, USA

Christine Goetz
Christopher Hammerbeck
Jody Bonnevier

Acknowledgments

We gratefully acknowledge Laura Prugar, Kim Stalbaum, Catherine Badger, Steve Orstad, Justin Haworth, and John Humphrey for critically evaluating this book.

We also would like to thank the Flow Cytometry Antibody Development group at R&D Systems for contributing data, providing critical discussion, and performing a number of laboratory tasks so we could devote our time to writing this book.

Thank you to Luke Huttner for recording, editing, and rendering the video for use in this book.

Thank you to Jody Bonnevier (Manager of Antibody Development) and Birte Aggeler (Director of Antibody Development and Flow Cytometry) at R&D Systems for giving us the freedom to write this book.

Finally, we would like to thank our families for all their support and patience during the process of writing this book.

Contents

1	Flow Cytometry: Definition, History, and Uses in Biological Research.	1
	Jody Bonnevier, Christopher Hammerbeck, and Christine Goetz	
1	Components of Flow Cytometry	1
2	Definition of Flow Cytometry	2
3	History of Flow Cytometry	4
3.1	Cellular Impedance and the Coulter Principle	4
3.2	Fluorescence-Based Flow Cytometers	5
3.3	Monoclonal and Polyclonal Antibodies	6
3.4	Fluorescent Molecules	7
3.5	Fluorescence Activated Cell Sorting (FACS)	7
4	Modern Flow Cytometers	8
4.1	Fluidic-Based Flow Cytometers	8
4.2	Acoustic-Based Flow Cytometers	9
4.3	Mass Cytometers (CyTOF)	9
	References	11
2	Physics of a Flow Cytometer	13
	Jody Bonnevier, Jae-Bong Huh, Christopher Hammerbeck, and Christine Goetz	
1	Components of Fluidic-Based Flow Cytometers	13
1.1	Fluidics	13
1.2	Optics	15
1.3	Electronics	20
2	Putting It All Together	22
3	Materials	23
3.1	Flow Antibodies	23
3.2	Cell Activation Reagents	24
3.3	Cell Purification Kits	24
3.4	Staining Buffers/Fc Block Reagents	24
	References	24

3	The Language of Flow Cytometry and Experimental Setup	25
	Christine Goetz and Christopher Hammerbeck	
1	Flow Data Plots and Gating	25
1.1	Flow Data Plots	25
1.2	Gating	27
2	Mean Fluorescence Intensity (MFI) and Biexponential vs. Logarithmic Scaling	27
2.1	Mean Fluorescence Intensity (MFI)	28
2.2	Logarithmic vs. Biexponential Scaling	28
3	Forward Scatter (FSC)/Side Scatter (SSC) and Doublet Exclusion	30
3.1	FSC/SSC	30
3.2	Doublet Exclusion	31
4	CS&T and Basic Experimental Setup	33
4.1	CS&T	33
4.2	Basic Experimental Setup	33
4.2.1	Experimental/Flow Panel Design	33
4.2.2	Surface and Intracellular Staining on iTregs	33
4.2.3	Experimental Setup on the Fortessa	34
4.2.4	Sample Acquisition and Analysis	34
5	Basic Concepts of Compensation	36
6	Materials	39
6.1	Flow Antibodies	39
6.2	Cell Activation Reagents	39
6.3	Cell Purification Kits	40
6.4	Staining Buffers/Fc Block Reagents	40
	References	40
4	Fluorochrome Choices for Flow Cytometry	41
	Christine Goetz and Christopher Hammerbeck	
1	Fluorochromes and Their Pros and Cons	41
2	Spectrum Viewers and How to Use Them	43
3	Fluorochrome Combinations to Use/Avoid Together	43
4	Laser Strength on the Fortessa vs. the Calibur	47
5	High- vs. Low-Density Cell Markers and Fluorochrome Choice	47
6	Materials	50
6.1	Flow Antibodies	50
6.2	Staining Buffers/Fc Block Reagents	51
	References	51
5	Using the Process of Compensation to Prevent False Positive Data Caused by Fluorescence Spillover: A Practical Example	53
	Christopher Hammerbeck, Christine Goetz, and Li Jen Peng	
1	Compensation: A Practical Example	53
2	Manual Compensation by Comparing the Median Fluorescence Intensity of Cell Populations	66
3	Considerations When Performing Compensation	69

3.1	Using the Same Compensation Settings for Multiple Experiments	69
3.2	Dimming of Fluorochromes as a Result of Compensation . . .	70
3.3	Consider the Number of Overlapping Fluorochromes	70
3.4	Consider If Compensation Is Necessary	71
3.5	Consider If Fluorochromes with High Spectral Overlap Can Be Separated on Two Different Cell Types	71
3.6	Using Cells as Single-Stain Controls vs. Beads	73
4	Materials	73
4.1	Flow Antibodies	73
4.2	Staining Buffers/Fc Block Reagents	74
	References	74
6	Primary and Secondary Antibodies and Flow Cytometry Controls	75
	Christopher Hammerbeck, Christine Goetz, and Jody Bonnevier	
1	Antibody Development for Flow Cytometry	75
2	Detection of Unconjugated Primary Antibodies	78
3	Flow Cytometry Controls	83
3.1	Isotype Controls	84
3.2	Internal Lineage Controls	88
3.3	Cell Activation Controls	92
3.4	Fluorescence Minus One (FMO)	96
4	Materials	100
4.1	Flow Antibodies	100
4.2	Isotype Control Antibodies	101
4.3	Secondary Antibodies	101
4.4	Cell Activation Reagents	101
4.5	Staining Buffers/Fc Block Reagents	101
	References	102
7	Experimental Considerations with Data Sets as Examples	103
	Christopher Hammerbeck, Christine Goetz, Li Jen Peng, and Jae-Bong Huh	
1	Fc Receptor Blocking	103
2	Dead Cell Exclusion	108
3	Cell Number, Antibody Concentration, and Titration of Flow Antibodies	114
3.1	Cell Number	114
3.2	Antibody Concentration	116
3.3	Titration of Flow Cytometry Antibodies	117
4	Surface Staining Considerations	119
4.1	From 2 to 12 Parameter Flow Cytometry	119
4.2	Reagents to Assess Cell Proliferation	121
4.3	Tracking Cell Populations in Mice Using Congenic Markers	125

4.4	Chemokine Receptor Staining and Dependence on Temperature	126
4.5	How to Detect Degranulation in NK Cells Using a Killing Assay	128
4.6	Protein Detection Using Fluorokines	132
4.7	Dump Channels	133
5	Intracellular Staining Considerations	135
5.1	Intracellular Staining Buffers	135
5.2	Detection of Secreted Cytokines	137
5.3	Optimizing Fixation, Staining Order, and Using the Correct Intracellular Buffer System	138
5.3.1	Optimizing Fixation Percentage	139
5.3.2	Staining Order Matters When Detecting Both Surface and Intracellular Markers	140
5.3.3	Intracellular Buffer Systems for Transcription Factors and Phospho-Proteins	141
6	Materials	145
6.1	Flow Antibodies	145
6.2	Isotype Control Antibodies	146
6.3	Proliferation and Viability Dyes and Reagents	147
6.4	Cell Activation Reagents	147
6.5	Cell Purification Kits	147
6.6	Staining Buffers/Fc Block Reagents	147
	References	148
8	Cell Enrichment	149
	Christine Goetz, Christopher Hammerbeck, Andrew Wyman, and Jae-Bong Huh	
1	Enrichment of Cell Populations Using Columns	149
2	Magnetic Selection Principles and Types of Selection	150
3	Fluorescence Activated Cell Sorting (FACS)	153
4	Materials	154
4.1	Flow Antibodies	154
4.2	Isotype Controls	154
4.3	Cell Purification Kits	155
4.4	Staining Buffers/Fc Block Reagents	155
9	Surface and Intracellular Staining Protocols for Flow Cytometry . .	157
	Christine Goetz and Christopher Hammerbeck	
1	Staining Buffers for Flow Cytometry	157
1.1	Key Tips for Surface and Intracellular Staining	158
2	Surface Staining: Tips and Tricks	159
2.1	Cell Surface Staining in Whole Blood (WB)	160
2.1.1	Cell Surface Staining for Unconjugated Antibodies in Human WB	160

2.1.2	Cell Surface Staining for Directly Conjugated Antibodies in Human WB	161
2.2	Cell Surface Staining in Peripheral Blood Mononuclear Cells (PBMCs)	162
2.2.1	Cell Surface Staining for Unconjugated Antibodies in PBMCs	162
2.2.2	Cell Surface Staining for Directly Conjugated Antibodies in PBMCs	163
2.3	Cell Surface Staining for Biotinylated Antibodies	164
2.3.1	Cell Surface Staining for Biotinylated Antibodies in WB	164
2.3.2	Cell Surface Staining for Biotinylated Antibodies in PBMCs	165
2.4	Cell Surface Staining for Chemokine Receptors	166
2.5	Cell Surface Staining for LAMP Proteins	167
3	Intracellular Staining: Tips and Tricks	168
3.1	Intracellular Staining: Formaldehyde/Saponin	168
3.1.1	Formaldehyde/Saponin IC Staining in WB with Unconjugated Antibodies	168
3.1.2	Formaldehyde/Saponin IC Staining in WB with Directly Conjugated Antibodies	170
3.1.3	Formaldehyde/Saponin IC Staining in PBMCs with Unconjugated Antibodies	171
3.1.4	Formaldehyde/Saponin IC Staining in PBMCs with Directly Conjugated Antibodies	173
3.2	Intracellular Staining: FoxP3/Transcription Factor Buffer Set	174
3.2.1	FoxP3/Transcription Factor Buffer Staining in WB with Unconjugated Antibodies	174
3.2.2	FoxP3/Transcription Factor Buffer Staining in WB with Directly Conjugated Antibodies	175
3.2.3	FoxP3/Transcription Factor Buffer Staining in PBMCs with Unconjugated Antibodies	176
3.2.4	FoxP3/Transcription Factor Buffer Staining in PBMCs with Directly Conjugated Antibodies	177
3.3	Formaldehyde/Methanol Intracellular Staining	178
3.3.1	Formaldehyde/Methanol Staining for Detection of Unconjugated Phospho-Proteins in PBMCs or Stimulated Cell Populations	179
3.3.2	Formaldehyde/Methanol Staining for Detection of Directly Conjugated Phospho-Proteins in PBMCs or Stimulated Cell Populations	180

10	Troubleshooting	183
	Christopher Hammerbeck and Christine Goetz	
1	What Happens If I Permeabilize My Cells Before Fixation?	
	What Happens If I Forget to Dilute My Perm Buffer to 1×?	183
2	What Happens If I Use PE or APC-Conjugated Surface	
	Markers Prior to Permeabilization with Methanol?	184
3	What Happens If I Stain My Cells with the Same Antibody	
	Used for Stimulation?	185
4	How Can I Avoid Messy Staining with Costains	
	and Secondary Antibodies of the Same Species?	187
5	Why I Am Not Seeing NK1.1 Expression on Balb/c Mouse	
	Splenocytes? Is CD14 Universally Expressed in Human and Mouse	
	Monocytes? How About Other T and B Lineage Subsets?	188
6	What Happens If You Use a FITC or A488 Antibody	
	on GFP-Tagged Cells?	189
7	Should I Use FcR Block on All Cells and with All Antibodies? . . .	190
8	Do I Need to Use PMA, Lonomycin, and Monensin	
	(and/or Brefeldin A) for Detection of Secreted Cytokines	
	in T Cells and Monocytes?	191
9	Can I Add My Secondary and Primary Antibody at the Same	
	Time to Speed Up an Experiment?	192
10	Can Biotinylated Antibodies and the Streptavidin Secondary	
	Be Added at the Same Time?	193
11	Does Volume Matter When Surface Staining Your Cells?	194
12	What Happens If I Can't Run My Cells on the Cytometer	
	on the Same Day that I Stain Them?	196
13	What Happens If I Accidentally Add the Wrong Antibody	
	to My Tube? Can I Quickly Wash It Out and Reuse	
	Those Cells?	196
14	Can Flow Cytometry Be Used to Count Cells?	198
15	Can the Method Used to Harvest Adherent Cells Affect	
	the Ability to Detect Protein Expression by Flow Cytometry?	199
16	Materials	200
	16.1 Flow Antibodies	200
	16.2 Isotype Control Antibodies	201
	16.3 Secondary Antibodies and Staining Reagents	202
	16.4 MISC Flow Cytometry Reagents	202
	16.5 Cell Culture Reagents	202
	16.6 Cell Activation Reagents	202
	16.7 Cell Purification Kits	202
	16.8 Staining Buffers	202
	References	203
	Glossary	205
	Index	213

About the Book

This first edition volume demystifies the complex topic of flow cytometry by providing detailed explanations and nearly 120 figures to help novice flow cytometry users learn and understand the bedrock principles necessary to perform basic flow cytometry experiments correctly.

The book divides the topic of flow cytometry into easy to understand sections and covers topics such as the physics behind flow cytometry, flow cytometry lingo, designing flow cytometry experiments and choosing appropriate fluorochromes, compensation, sample preparation and controls, and ways to assess cellular function using a variety of flow cytometry assays. Written as a series of chapters whose concepts sequentially build on one another, using the list of materials contained within each section along with the readily reproducible laboratory protocols and tips on troubleshooting that are included, readers should be able to reproduce the data figures presented throughout the book on their way to mastering sound basic flow cytometry techniques.

Easy to understand and comprehensive, *Flow Cytometry Basics for the Non-Expert* will be a valuable resource to novice flow cytometry users as well as experts in other biomedical research fields who need to familiarize themselves with a basic understanding of how to perform flow cytometry and interpret flow cytometry data. This book is written for both scientists and nonscientists in academia, government, biotechnology, and medicine.

Chapter 1

Flow Cytometry: Definition, History, and Uses in Biological Research



Jody Bonnevier, Christopher Hammerbeck, and Christine Goetz

Flow cytometry was developed based on a need to analyze protein expression and phenotype live cells. Proteins expressed on the cell surface are referred to as surface markers in this field. These protein and protein combinations are what give the cells their unique phenotype, against which, specific antibodies are developed and used to identify populations of cells expressing these proteins. Visualizing and identifying cells by microscopy was not enough; a way to quantify phenotypic differences in live cell mixtures based on biological markers was needed as scientists asked questions about specific cell populations in a heterogeneous mixture, such as peripheral blood. In addition to identifying cells, mixed populations of live cells needed to be separated, or sorted based on phenotype, and so cell sorting was born. Flow cytometry is composed of three essential components: instrumentation, monoclonal antibodies, and fluorochrome. The co-evolution of the flow cytometry instrumentation, and sensitive and specific antibodies together with fluorescent dyes has driven this technology to become the mainstay in every type of biomedical and clinical research and patient testing imaginable.

1 Components of Flow Cytometry

1. *Flow cytometer instrument*: Fluidics-based flow cytometers are composed of a fluidics system designed to move cells through the instrument, one or more lasers to excite fluorochromes, an optics system to detect light emitted by the excited fluorochromes, and software to translate the collected data into a

J. Bonnevier · C. Hammerbeck · C. Goetz (✉)
Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA
e-mail: Jody.Bonnevier@bio-techne.com; Chris.Hammerbeck@bio-techne.com; Christine.Goetz@bio-techne.com

user-friendly readout. The history of flow cytometry instrumentation will be covered in this chapter. The workings of this instrumentation will be covered in more detail in Chap. 2.

2. *Antibodies for flow cytometry*: The availability of robust, specific, and sensitive antibodies is critical to the success of any flow cytometry experiment. In contrast to antibodies needed for Western blot, which detect fully denatured, linear epitopes, antibodies for flow cytometry are required to detect proteins in a natural, fully folded conformation on a live or fixed cell. Antibodies are generated by immunization of animals with antigens. Often the antigens are peptides, but using full-length, active, recombinant proteins as immunogens can produce antibodies that are generally skewed toward detection of natural conformation protein epitopes on cells. There is a critical need for any antibodies used in flow cytometry experiments to be rigorously validated and characterized using various methods. The antibody validation process for flow cytometry can include ELISA, Western blot, Immunocytochemistry (ICC), Immunohistochemistry (IHC), biological cell models, transfected cell lines, knockouts, and orthogonal methods, which will be discussed in more detail in Chap. 6.
3. *Fluorescent dyes*: Flow cytometry would not be feasible without a large number of fluorescent dyes conjugated to antibodies. Many choices are available that enable multiplexing and we will go over how to choose from the rainbow of colors in Chap. 4.

2 Definition of Flow Cytometry

What is flow cytometry? The term can be broken down into three parts to provide a definition:

Flow = fluidic, **cyto** = cell, and **metry** = measure.

In other words, flow cytometry is the measure or quantification of cells suspended in a fluid phase. The cells are labeled with fluorochrome-coupled antibodies specific for a cellular marker of interest. The fluidics chamber ensures that cells pass single file through a laser beam, which excites the fluorochrome. The emitted light is picked up by a detector (photomultiplier), and the signal is translated electronically to a computer for analysis (Fig. 1.1). Because of its single-cell nature, flow cytometry allows for the analysis of protein expression on a per cell basis, making it quantifiable with results expressed as a count or number of events. The technique allows researchers to evaluate and quantitate specific cell types within a heterogeneous population enabling the analysis of small numbers of cells or rare subsets in a mixed population. It also allows for multiprotein analysis within a single cell when using multiple antibodies conjugated to different fluorescent dyes. This makes flow cytometry a prevalent technique that today is used in nearly every aspect of cell biology research and clinical patient cell analysis.

The strength of flow cytometry in analysis of mixed cell populations, such as subsets of human peripheral blood cells, is illustrated in Fig. 1.2. In a homogenous

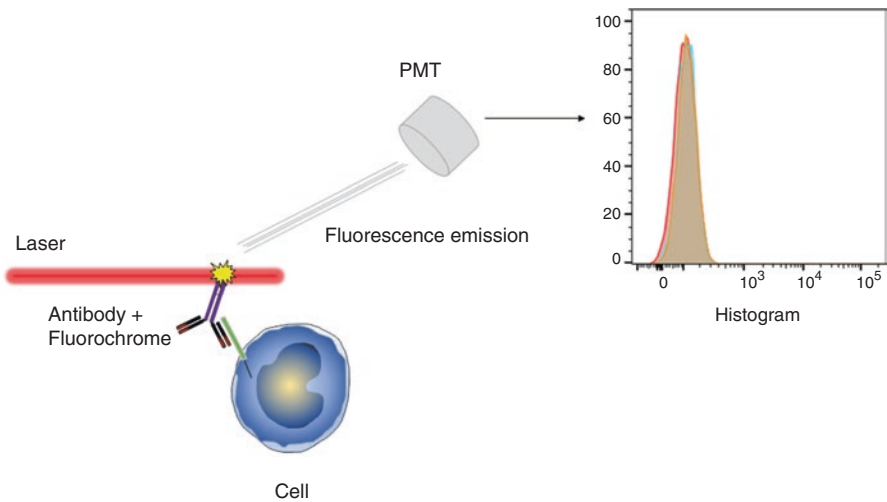


Fig. 1.1 Flow cytometry overview. Fluorochrome-conjugated antibody binds to a protein of interest on a cell. The cell passes through the laser beam, which excites the fluorochrome and emits light of a certain wavelength. This emission is detected by the instrument and translated by the software to a histogram or other quantitative representation

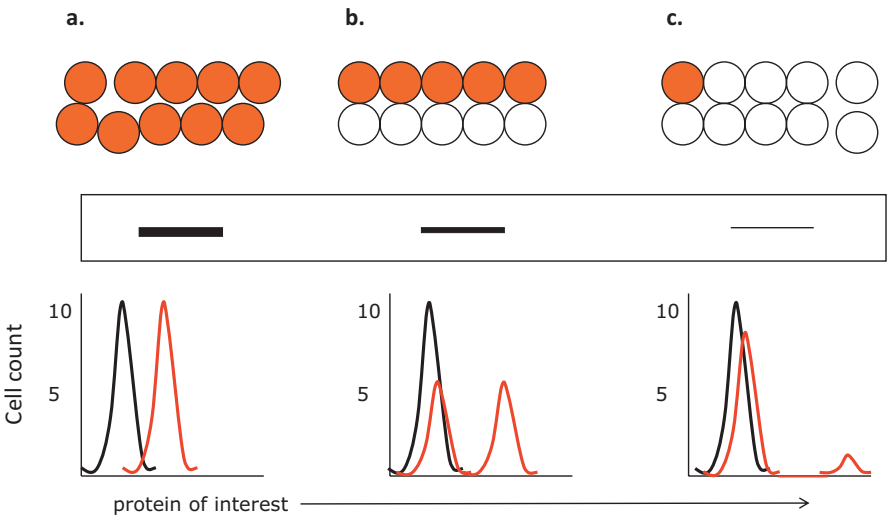


Fig. 1.2 Flow cytometry analysis of mixed cell populations. Flow cytometry versus Western blot analysis of a (a) homogeneous population of cells, (b) a heterogeneous mix of cell types, and (c) a small subset of cells in a heterogeneous mix. Cell expressing the desired protein are colored orange

cell sample (i.e., cell line), most cells will express an equivalent amount of the protein of interest. A Western blot used to determine expression will detect one band, and flow cytometric analysis of the cells will result in a single peak (orange, the

black histogram is a negative control peak). In Fig. 1.2a, there are 10 cells that uniformly express the protein of interest resulting in a single Western blot band and peak in the flow plot. In a 50/50 mix of two distinct cell types (Fig. 1.2b), five cells express the protein of interest, and five do not. A single band is still detected in the bulk Western blot lysate, but two distinct peaks of equal height are now visible in the flow plot. If a rare subset of cells (10% of the population) is analyzed (Fig. 1.1c), again, one single band will be seen in a Western blot, but the protein expression on the rare subset of cells is detected as a distinct, albeit small, peak in flow cytometry. The disadvantage of a Western blot is that you cannot determine the level of protein expression on all cell populations; is the band the result of all cells expressing the same amount of protein or a subset of cells expressing a high level of protein? When the expression of the protein of interest is equivalent on all cells, there is a direct correlation between the density of the Western blot band and the height of the peaks of the flow plots. Flow cytometry will distinguish between all cells expressing a low level of the protein versus a subset of cells expressing a high level of the protein. This illustration shows the effectiveness of flow cytometry for the detection of rare subsets of cells in mixed populations. Immunohistochemistry (IHC) or Immunofluorescence (IF) microscopy can also be used to examine protein expression (in tissues rather than cells) but has limitations in the number of antibodies that can be screened for subset analysis and is less quantitative. The advantage of IHC or IF is that you can visualize protein expression in various tissues and examine cellular distribution.

3 History of Flow Cytometry

3.1 Cellular Impedance and the Coulter Principle

Flow cytometry originated based on the principle of cellular impedance, a system for counting cells using the Coulter principle, patented by Wallace H. Coulter in 1953 [1, 2]. The principle states that in a fluid phase, the size of a cell may be determined by the volume of electrolyte it displaces as it passes through a pair of electrodes. In other words, if there is a current passing between electrodes and a cell passes through them, it temporarily interrupts, or impedes, the current. The length of time of the interruption is directly proportional to the size of the cell, so a small cell (red blood cell), would impede the current less than a large cell (neutrophil). The displaced volume is translated into a voltage pulse, which is then detected by an oscilloscope, and later by a computer. As shown in Fig. 1.3, two electrodes have an electrical current between them (wavy lines). A small cell disrupts this current temporarily, and the time of the disruption is proportional to the size of the cell. A larger cell displaces the current for a longer time. This technique can detect cells of different shapes as well as sizes, and two cells moving through the channel together will result in a double peak.

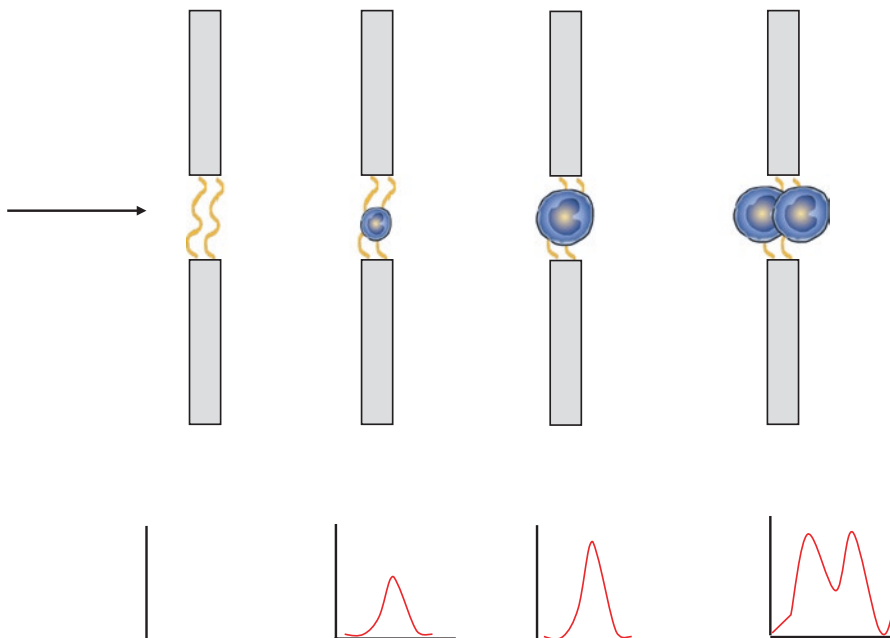


Fig. 1.3 Cellular impedance based on the Coulter Principal. Two electrodes have an electrical current between them. This electrical current is disrupted, or impeded, when cells pass between them. This impedance is detected by the instrument, and recorded as a histogram, indicating the size of the cell (histogram height, y -axis), or when two cells move through the current together rather than in a single file (histogram modality, x -axis)

Mack Fulwyler next built on the Coulter principle to develop an instrument used to sort cells [3]. Different-sized cells in droplets moved through an electrostatic field, and the difference in size was used to sort cells into collection bins. Although many advances have been made in cell sorting, the same technique of directing cell-containing droplets through an electric field remains in use today. Cell sorting will be discussed later and in Chap. 8.

3.2 Fluorescence-Based Flow Cytometers

The first fluorescence-based flow cytometer was developed in 1968 by Wolfgang Göhde at the University of Münster, and sorted cells based on fluorescence rather than cell size [4]. One of Göhde's most important contributions to the field of cytometry was development of the flow cell, which allowed for cells to be channeled into a focal point in order to be analyzed individually. Another important advancement was the use of fluorescent molecule emission rather than absorption, which was more favored at the time. The flow cytometer was first commercialized in 1968 by the German company Partec, soon after followed by instruments from Bio/Physics Systems (Cytofluorograph, 1971), and Partec (PAS 8000, 1973).

3.3 *Monoclonal and Polyclonal Antibodies*

The introduction of fluorescence-based flow cytometers necessitated the development of the other two critical components of flow cytometry: monoclonal antibodies and fluorescent molecules. Before the advent of monoclonal antibodies, polyclonal antibodies were used exclusively. Polyclonal antibodies are a group of antibodies that bind to multiple epitopes of the same target antigen produced by multiple B cells. One major advantage of polyclonal antibodies is their high affinity due to the nature in which they are made. Whole proteins are used in the immunization process allowing for detection of multiple epitopes. These antibodies are less sensitive to fixation as multiple epitopes are available for detection. Two clear disadvantages include antibody variability and higher potential for cross reactivity. Instead of establishing immortalized hybridomas that produce monoclonal antibodies, polyclonal antibodies are typically generated in larger animals (goats, sheep, cows, and horses) and the antibodies are purified from the serum of these animals. This means that a particular batch of polyclonal antibodies is available only for the lifespan of the animal and batch-to-batch variability can exist as a result of slight genetic differences between individual donor animals. Since whole proteins are typically used to generate polyclonal antibodies, cross-reactivity between different isoforms of the same protein can exist, at times making a polyclonal antibody less specific than a monoclonal antibody.

In 1975, George Kohler and Cesar Milstein made the breakthrough discovery of hybridoma technology [5]. In the body, normal B cells make antibodies in response to exposure to a foreign antigen, but stop making them when the infection is cleared. Kohler and Milstein found that by taking that antibody-producing B cell and fusing it with a cancerous myeloma cell they could produce a hybridoma cell that continuously makes unlimited amounts of antibody. This results in the production of a monoclonal antibody, which can be defined as an antibody that has monovalent affinity that binds a single epitope of the same target antigen. In contrast to polyclonal antibodies, monoclonal antibodies can be produced in large quantities with high lot-to-lot consistency and high specificity to a single epitope. However, this high specificity to a single epitope also renders monoclonal antibodies more sensitive to changes in the epitope structure making it less robust for detecting the protein in a denatured state or altered conformation. In this way, the door was opened for the development of large quantities of specific antibodies used to label cells of interest in flow cytometry and other applications, and accordingly, the Nobel prize was awarded in 1984 for this significant advancement. More recently, recombinant monoclonal antibody technology has been developed and does not rely on hybridomas. Rather, monoclonal antibody genes are recovered from B cells, amplified and cloned into the appropriate vector system to allow for unlimited production of the antibody by bacterial or other mammalian cell lines.

3.4 *Fluorescent Molecules*

The “hour of the fluorescent antibody” began in 1942 [6]. Initially, Coons and colleagues after struggling with anthracene isothiocyanate turned to labeling anti-Pneumococcus antiserum (strain 3) with fluorescein iso-cyanate more commonly known as FITC. They found they could detect fluorescent staining when labeling tissues infected with strain 3, but not strain 2 Pneumococcus, demonstrating one of the first uses of an antigen-specific fluorescently tagged antibody [7]. Fluorochromes are fluorescent chemical compounds that emit light upon excitation at a distinct wavelength. There are many protocols and chemistries available for conjugating fluorochromes to antibodies, and the Molecular Probes Handbook is an excellent in-depth resource [8]. The most commonly used chemistry for conjugation is the use of amine-reactive probes. The three major types of reagents used to label amines are active esters, isothiocyanates, and sulfonyl chlorides. Active esters are preferred since they produce stable carboxamide bonds between the dye and the antibody [9]. In traditional flow cytometry, fluorochromes are excited with laser light of a specific wavelength. The excited fluorophore then emits light at a specific emission wavelength, which is detected by the photomultiplier tube in the cytometer.

As experimental questions and cytometry experiments get more and more complex, the need for a larger number of fluorochromes, detected simultaneously using multiple absorption/emission combinations, becomes a limiting factor. Development of brighter, more stable fluorochromes is always in the forefront of flow cytometry development. A more thorough description of how these fluorochromes work can be found in Chap. 2 and how to wisely choose these fluorochromes will be discussed in Chap. 4.

3.5 *Fluorescence Activated Cell Sorting (FACS)*

Leonard Herzenberg quickly recognized the need to build on the idea of live cells being sorted based on fluorescence. To accomplish this, monoclonal antibodies specific to markers on the cells of interest coupled to fluorescent molecules allowed for fluorescent sorting of cells based on the phenotype of surface markers, a technique the Herzenberg lab pioneered [10]. Dr. Herzenberg coined the word “FACS” or **F**luorescence **A**ctivated **C**ell **S**orting to describe the method in which fluorescence, rather than size, is used to sort cells [11]. The term FACS is often misconstrued to refer to collecting cells on a flow cytometer and visualizing them on multicolor flow plots where it is really the physical separation of cells based on electrical charge and antibody fluorescence. The first FACS instrument had one laser and two light detectors, one to measure cell size, and one to measure fluorescence [12]. The first commercial FACS instrument was developed by Becton Dickinson (BD) Biosciences (1974), and the Partec/Phywe (ICP 22, 1975), and Epics from Coulter (1977) followed shortly after.

4 Modern Flow Cytometers

There are three types of flow cytometers available on the market today:

1. Fluidic-based flow cytometers.
2. Acoustic-based flow cytometers.
3. Mass cytometers or CyTOF.

Fluidics-based flow cytometers are by far the most widely used, and the number of laser/detector parameters is always increasing. Acoustic-based cytometers use acoustic focusing rather than fluidics. Mass cytometry utilizes monoclonal antibodies conjugated to heavy metals rather than fluorochromes and can greatly increase the number of parameters analyzed simultaneously. Regardless of the type of flow cytometer that is being utilized, cells need to be labeled with antibodies conjugated to either fluorochromes (Fluidic and Acoustic-based flow cytometers) or in recent developments, heavy metal tags (mass cytometers), to detect protein expression on cells. Next we will go over these three types of flow cytometers in order to better understand the pros and cons of each system.

4.1 *Fluidic-Based Flow Cytometers*

Fluidics-based flow cytometers are highly utilized in research labs around the world. As flow cytometers have evolved, more lasers and complex optics have been added, thus allowing for more parameters to be analyzed simultaneously. We will discuss the mechanics of how fluidic-based flow cytometers work in Chap. 2. Initially, flow cytometers had one or two lasers—the blue laser (488 nm) and red laser (633 nm). The blue laser can excite fluorochromes such as Fluorescein Isothiocyanate (FITC), Phycoerythrin (PE) and Peridinin-chlorophyll Protein Complex (PerCP), and the red laser can excite Allophycocyanin (APC). Photomultiplier detection (see Chap. 2 for more details) of these four fluorophores formed the basis for flow cytometry experiments in the early 1990s. Addition of the violet laser (405 nm) and the UV laser (350 nm) in the early 2000s opened up a previously unused portion of the light spectrum, and paved the way for development of new dyes excitable at these wavelengths. Recent addition of a fifth laser, the yellow-green (561 nm), allows for excitation of PE by a different laser than used to excite FITC, greatly reducing the need for compensation of spectral overlap of these two fluorochromes (discussed in detail in Chap. 5). Fluidic-based flow cytometers are advantageous to researchers due to their low cost and availability.

4.2 *Acoustic-Based Flow Cytometers*

The Attune NxT is an acoustic-based flow cytometer designed by Life Technologies. The instrument uses acoustic focusing with ultrasonic radiation pressure to transport cells to the center of the sample stream, which is then injected into the sheath stream. Using this technology, a narrow core stream and uniform laser illumination can be achieved (www.LifeTech.com). As a result, the cytometer is less prone to clogs and can analyze up to 35,000–50,000 events/s whereas fluidic-based cytometers are normally run at closer to 3000 events/s. The Attune can handle up to 14 parameters and autosamplers are available for running up to 384 samples using a plate-based format. As the detection of rare events such as tumor cells in whole blood becomes increasingly important in medical diagnostics and treatment, high analysis flow rates using acoustic flow cytometry can aid in detecting these low frequency cell populations. These machines are higher in price so they may be cost prohibitive for some researchers.

4.3 *Mass Cytometers (CyTOF)*

The Helios™ CyTOF (Cytometry by Time of Flight) from Fluidigm combines the best of flow cytometry and mass spectrometry. Introduced in 2014, CyTOF utilizes monoclonal antibodies labeled with heavy metal tags rather than fluorochromes (see the highlighted elements in the periodic table; Fig. 1.4). After labeling, the cells are sprayed as single droplets in an argon plasma (5000 °C), which are then vaporized, and the electrons are stripped from the metals to generate ions. The ions are accelerated into an electrostatic field and a deflector separates them as they fly toward the collecting screen at different rates designated by their mass (Fig. 1.5; schematic is based off the illustration on the Fluidigm website). The detector quantifies the ions and the data is analyzed and displayed on the computer. Sorting is not possible using this mass cytometer as the samples are incinerated; however, it has the ability to analyze 50–100 markers at a time. Whereas correction of spectral overlap is required in fluidic/acoustic-based flow cytometry, CyTOF requires little compensation due to the use of heavy metal tags instead of fluorochromes. Flow rates are much slower (500 events/s) than either fluidic-based or acoustic-based flow cytometers (3000 and 35,000, respectively). In addition, the Helios™ CyTOF cytometers are quite expensive. When this is taken into account along with the added cost of conjugating antibodies to heavy metal tags, this technology is cost prohibitive for most researchers. This technology is most useful in clinical settings where a large number of variables can be analyzed on a small sample size.

Flow cytometry has revolutionized the ways that scientists study, phenotype, and sort cells for further analysis. The ability to measure multiple parameters on a single cell is the most unique aspect of flow cytometry. Flow cytometry can be used for a wide range of applications, most commonly the detection of proteins on the surface

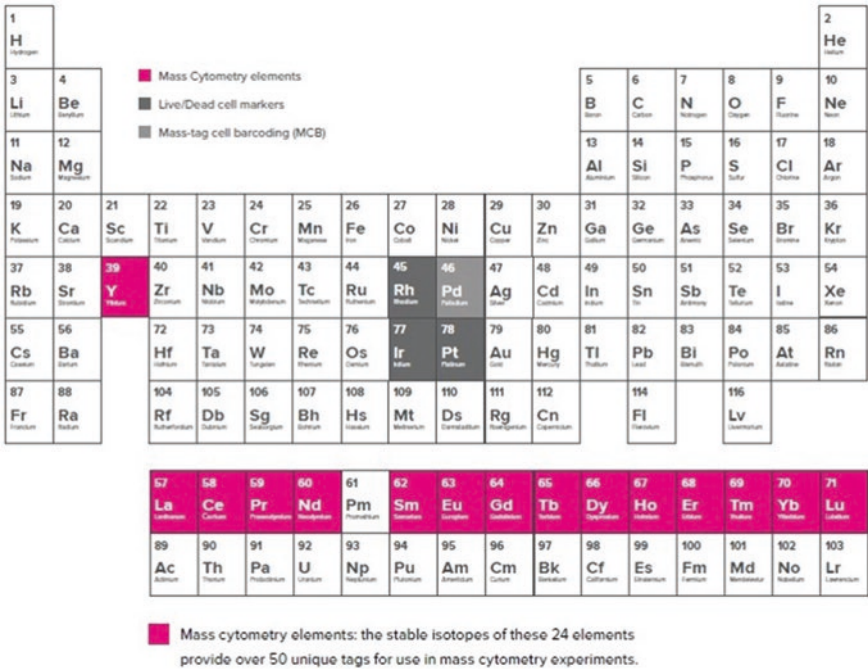


Fig. 1.4 Mass cytometry (CyTOF). Periodic table highlighting the metals used by Fluidigm™ to label antibodies in CyTOF (<http://www.fluidigm.com>)

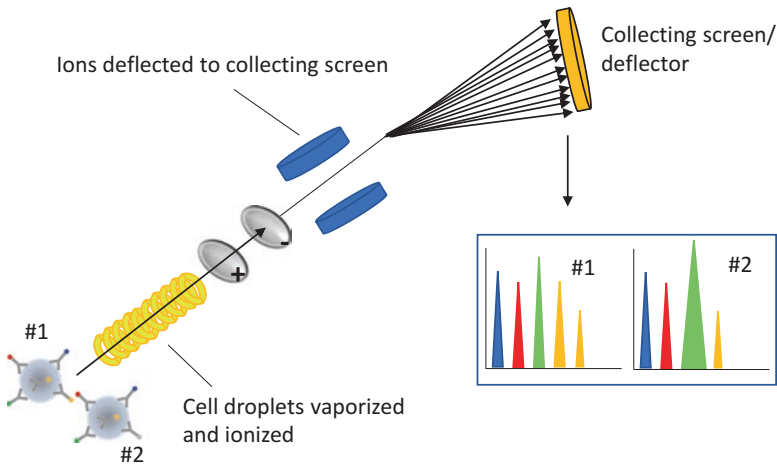


Fig. 1.5 Physics of the Helios™ CyTOF from Fluidigm™. Schematic illustrating how the CyTOF works. Briefly, cells labeled with antibodies conjugated to heavy metals are vaporized and ionized, the ions are then accelerated in an electrostatic field, separated by a deflector, and are quantified based on their mass. See the text for more details

of, or inside cells. Cell surface proteins can be detected on live cells, but cells can also be fixed and permeabilized to detect intracellular proteins such as cytokines, signaling molecules, and transcription factors. Flow cytometry can also be used to analyze DNA or RNA content [13]. It can be used to analyze viability, cell death and apoptosis, and cell cycle. Mammalian cells can be analyzed, but also insect cells, bacteria, isolated nuclei, and even exosomes. As the technology continues to expand, so will the uses for flow cytometry.

References

1. Coulter WH. Means for counting particles suspended in a fluid. United States Patent US2656508A. 1953.
2. Silveira GF. Evolution of flow cytometry technology. *J Microbial and Biochemical Technol.* 2015;7:213–6.
3. Fulwyler MJ. Electronic separation of biological cells by volume. *Science.* 1965;150:910–1.
4. Dittrich WM, Gohde WH. Flow-through chamber for photometers to measure and count particles in a dispersion medium. United States Patent US3761187A. 1968.
5. Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature.* 1975;256:495–7.
6. Coons AH. The beginnings of immunofluorescence. *J Immunol.* 1961;87(5):499–503.
7. Van Ooij C. Recipe for fluorescent antibodies. *Nat Milestones.* 2009;11:S10–1.
8. Johnson I, Spence MTZ. The molecular probes handbook: a guide to fluorescent probes and labeling technologies. 11th ed. California: Life Technologies Corporation; 2010.
9. Banks PR, Paquette DM. Comparison of three common amine reactive fluorescent probes used for conjugation to biomolecules by capillary zone electrophoresis. *Bioconjug Chem.* 1995;6:447–58.
10. Herzenberg LA, Parks D, Sahaf B, Perez O, Roederer M, Herzenberg LA. The history and future of the fluorescence activated cell sorter and flow cytometry: a view from Stanford. *Clin Chem.* 2002;48:1819–27.
11. Julius MH, Masuda T, Herzenberg LA. Demonstration that antigen-binding cells are precursors of antibody-producing cells after purification with a fluorescence-activated cell sorter. *Proc Natl Acad Sci U S A.* 1972;69:1934–8.
12. Herzenberg LA, Sweet RG, Herzenberg LA. Fluorescence-activated cell sorting. *Sci Am.* 1976;234:108–17.
13. Hanley MB, Lomas W, Mittar D, Park E. Detection of low abundance RNA molecules in individual cells by flow cytometry. *PLoS One.* 2013;8(2):e57002.

Chapter 2

Physics of a Flow Cytometer



Jody Bonnevier, Jae-Bong Huh, Christopher Hammerbeck,
and Christine Goetz

1 Components of Fluidic-Based Flow Cytometers

1.1 Fluidics

The fluidics system is an integral part of a flow cytometer. It is responsible for transporting the cells labeled with fluorescent antibodies to the interrogation point so they can be excited by the lasers. This is accomplished using hydrodynamic focusing by creating laminar flow, which is when two streams of fluid with different flow rates run side by side in the same direction (Fig. 2.1). Hydrodynamic focusing uses differential fluid flow (cell sample stream versus sheath fluid stream) rates to align cells single file in the flow cell of a flow cytometer. In the center stream, cells enter the flow cytometer randomly distributed in the sample fluid. The slower sheath fluid stream runs parallel to the sample stream. This creates laminar flow and focuses the cells in the flow cell nozzle so that they move through the flow cell one at a time to achieve single-cell resolution [1]. It is important that the cells are in a single stream so the lasers can optimally excite the fluorochrome-conjugated antibodies on the cells one cell at a time to achieve maximal resolution. The flow cell is the location within the cytometer where the cells are hydrodynamically focused into a single-cell stream.

While the flow rate of the sheath fluid stream remains constant, the flow rate of the sample stream may be adjusted to acquire samples at an optimal 3000 events per

J. Bonnevier · J.-B. Huh · C. Hammerbeck · C. Goetz (✉)
Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA
e-mail: Jody.Bonnevier@bio-techne.com; Jae-Bong.Huh@bio-techne.com;
Chris.Hammerbeck@bio-techne.com; Christine.Goetz@bio-techne.com

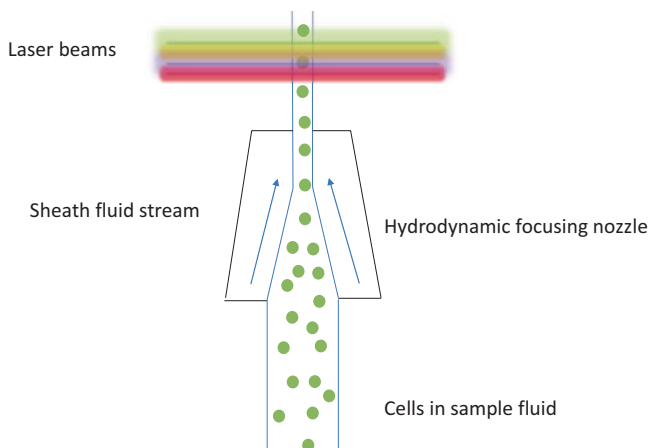


Fig. 2.1 Hydrodynamic focusing. Two streams of fluid—cells in sample fluid and sheath fluid—move side by side into the flow cell creating a laminar flow, which focuses the cells in the flow cell nozzle forcing them into single file

second. If the flow rate is too high, the stream may widen, allowing too many cells in at once. This is disruptive both to the single-cell resolution of the flow cytometer, and may also cause clogging of cells in the nozzle, with a possibility for sample loss. Cells that are concentrated will require a lower flow rate and cells that are dilute will require a higher flow rate. Adjusting the sample volume can aid in this process. In addition, cells should be filtered if clumps are visible. This step is crucial for flow cytometers to achieve single-cell resolution [2]. When analyzing samples using flow cytometry, most often than not, it is important to run the samples at lower flow rates. The low differential pressure is ideal for sorting and cell cycle analysis, as well as obtaining better resolution and better doublet discrimination. As shown in Fig. 2.2, running samples at too high a flow rate can increase your abort rate compared to running the same sample at a lower flow rate. When samples are acquired too quickly or at 6368 events/s (Fig. 2.2 top panel), the abort rate is around 484 events/s. In contrast, running the samples at a more optimal flow rate or 2388 events/s, results in a much lower abort rate of 64 events/s (Fig. 2.2 bottom panel). Abort rates are defined as events that are discarded by the flow cytometer when samples are being acquired too quickly to allow discrimination of real cellular events versus debris. If your abort rate is at 50% of the acquisition rate, you are literally throwing away half of your cells and they are not being analyzed. Furthermore, doublet discrimination is key when analyzing rare subpopulations, by reducing the amount of cells that are “kicked out” of the analysis and improving the efficiency of the data being collected.

The threshold can also be used to discriminate any unnecessary artifacts inherent in the process. This can be defined as a setting used to digitally filter out any artifacts that are not cellular events during acquisition based on minimum size. Many of the buffers used in flow cytometry are PBS-based. Over time this can result in

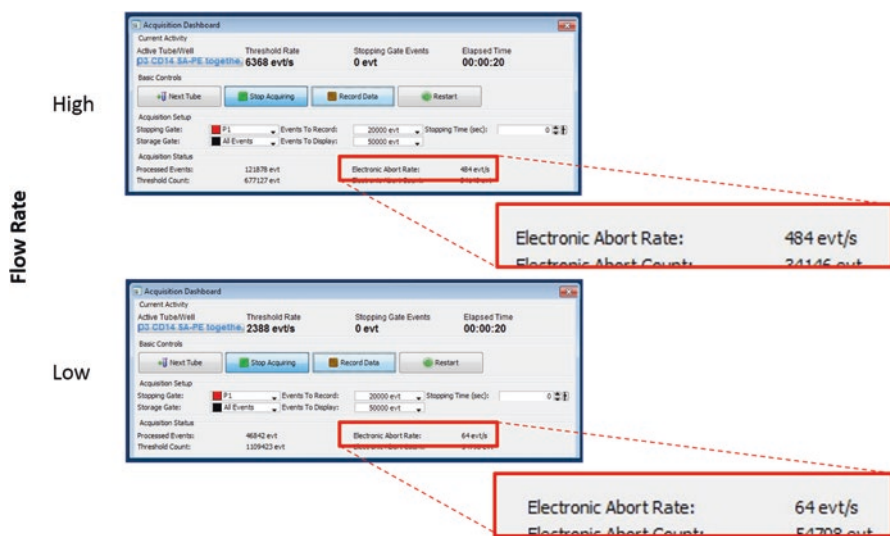


Fig. 2.2 Abort rates. Abort rates shown on the BD LSRFortessa™ when the cells are run on high (top panel) and low (bottom panel). The abort rates are highlighted in red boxes

artifacts which may show up in scatter plots. Also, many of the biological fluids analyzed via flow cytometry may contain unwanted artifacts and debris that can further convolute the data analysis. By setting a threshold, we can digitally filter out any of these artifacts, so that we are not recording unwanted data, which is particularly important when analyzing rare subpopulations.

1.2 Optics

After hydrodynamic focusing, the cells move in single file into the optics system (Fig. 2.1). The optics system is comprised of the Excitation Optics system, consisting of laser beams to scatter light and to excite the fluorochrome-labeled antibodies on cells, and the Collection Optics system, consisting of filters, and mirrors to direct the scattered and emitted fluorescent light to a series of Photomultiplier Tubes (PMTs). PMTs are also known as detectors or channels, which digitally convert the light into data points that are displayed in a graphical form in flow plots on the computer (Fig. 2.3). Photodiodes are less sensitive to light signals than PMTs and are used to detect the stronger Forward Scatter Channel. Side Scatter and fluorescence are weaker, thus PMTs required to detect these signals. Light that is scattered forward is collected by the Forward Scatter Channel (FSC), and is used as a gauge of cell size, but also takes into account nuclear to cytoplasmic ratio, membrane topography, and other cellular characteristics. Light that is scattered perpendicular to the laser beam is detected as Side Scatter (SSC), and provides information about cellular granularity and complexity.

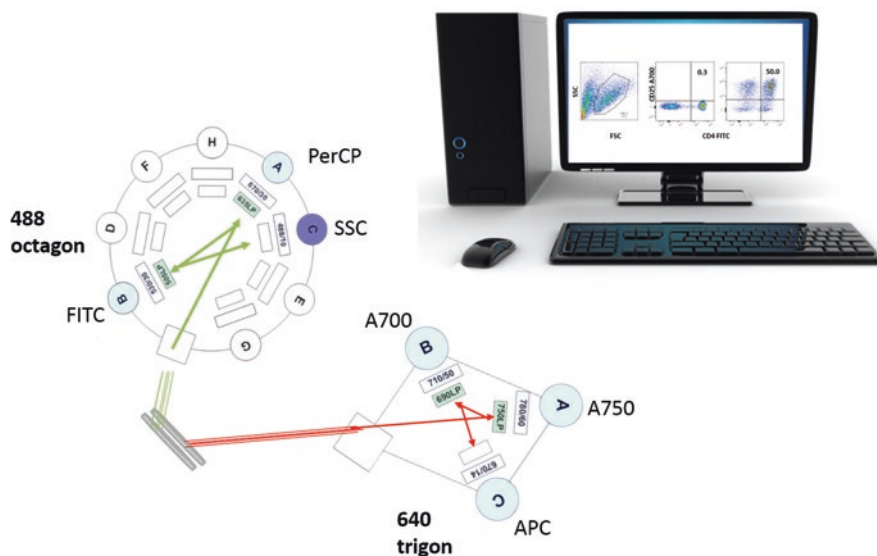


Fig. 2.3 Optics and Electronics. The optics system filters emitted light and directs it to the electronics system for translation to software for data readout (BD LSRFortessa™ cytometer configuration window)

A dot plot comparing forward (FSC) and side (SSC) light scatter is usually the starting point for flow cytometry analysis and gives valuable information about the cells. A heterogeneous mixture of peripheral blood cells detected in the FSC and SSC channels is shown in Fig. 2.4. Using only these two parameters, in the absence of any antibody staining for phenotypic markers, it is quite easy to evaluate and separate the different cell types in the mixed sample. To focus on the cell subsets, circles, or “gates” are drawn around each of the cell types. Lymphocytes (T cells, B cells, NK cells) are relatively small. Monocytes are slightly larger and more granular, and granulocytes (neutrophils, eosinophils) are detected highest in FSC and SSC. Cells in the lower left corner are very small cells such as red blood cells, platelets, dying or dead cells, and cell debris. In many cases, cell debris can be eliminated from flow cytometry analysis by adjusting the threshold setting to ignore these extraordinarily small-sized events. This feature is optional as cell debris can be, and are typically, gated out during conventional post-acquisition data analysis but including cell debris events will increase the byte size of the respective flow cytometry data files.

In addition to the information gathered by the FSC and SSC channels, the optics system is responsible for filtering and directing the fluorescent light emitted by fluorochromes to the PMTs specific for those fluorochromes. Each cytometer contains a series of excitation lasers that emits a beam of light at a specific wavelength. Early

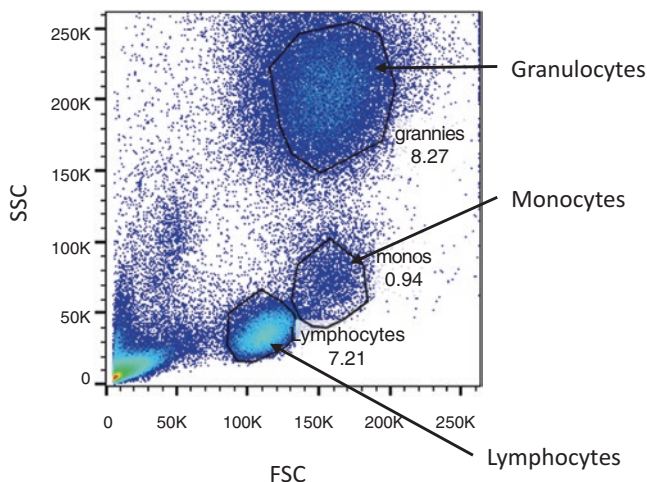


Fig. 2.4 Forward scatter (FSC) and Side scatter (SSC) show cellular characteristics. Cells passing through the laser deflect light forward to indicate cell size, and perpendicular to indicate granularity. In this example, FSC $\times$ SSC of human whole blood shows that different types of peripheral blood cells can easily be analyzed based on their light scatter properties

flow cytometers such as the BD FACScan™ had a single 488 nm (blue) excitation laser that emitted a beam of light at a wavelength of 488 nm. That instrument had three corresponding PMTs named FL1, FL2, and FL3 to detect light by fluorochromes excited by the 488 nm wavelength laser. More modern flow cytometers such as the BD LSR Fortessa™ can contain up to five excitation lasers: a 350 nm laser, a 405 nm laser, a 488 nm laser, a 561 nm laser, and a 640 nm laser. Each laser produces light at one specific wavelength: 350 nm, 405 nm, 488 nm, 561 nm, and 640 nm. Each laser is also commonly referred to by the color of light that it emits based on the visible light spectrum. For example, the 405 nm laser emits light at 405 nm. 405 nm light is within the violet color spectrum (380–450 nm) so the 405 nm laser is commonly referred to as the violet laser. As such, 350 nm light is within the ultraviolet light spectrum (10 nm–380 nm) so the 350 nm laser is referred to as the ultraviolet laser; 488 nm light is within the blue light spectrum (450 nm–495 nm) so the 488 nm laser is referred to as the blue laser; 640 nm light is within the red light spectrum (620 nm–750 nm) so the 640 nm laser is referred to as the red laser. 561 nm light falls between the green and yellow light spectrums (495–570 nm and 570–590 nm) so the 561 nm laser is referred to as the yellow-green laser.

As shown in Fig. 2.5, within the flow cell of the cytometer, the laser beams from each excitation laser are aligned such that the beams run parallel to each other. The excitation wavelength of a fluorochrome is specific to particular lasers that will excite it. Although many compounds can produce light when excited by lasers of various wavelengths, to be of practical use for flow cytometry, fluorochromes that are conjugated to flow cytometry antibodies need to possess excitation wavelengths that closely match the wavelengths of the light emitted by the lasers contained

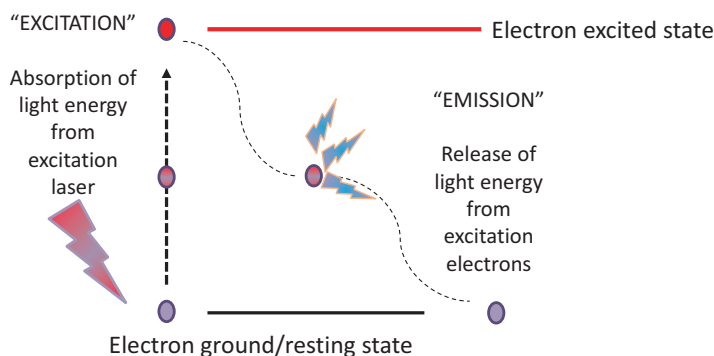
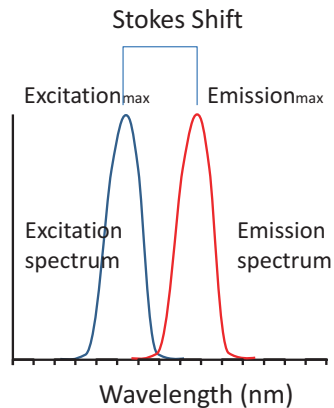


Fig. 2.6 Jablonski diagram. Resting electrons absorb light energy from the excitation laser and enter into an excited state for a very short period of time. As excited electrons return to a resting state, they emit photons of light. Refer to the text for more details

Just as fluorochromes absorb emitted light within a range of wavelengths, the photons of light that are emitted by the fluorochrome molecules have a range of wavelengths that are specific to each fluorochrome. Typically, the excitation wavelengths (in nanometers) for a particular fluorochrome are lower (i.e., higher energy) than the emission wavelengths for that same fluorochrome (higher wavelength in nanometers; lower energy). When excited, FITC emits photons of light between 475 nm and 650 nm with a peak emission wavelength of 525 nm. Because fluorochromes are excited by and emit light at a range of wavelengths, it is easier to characterize fluorochromes by wavelengths of light by which they are maximally excited and at which they maximally emit light. These values are called the excitation maximum (Excitation_{max} or EX_{max}) and emission maximum (Emission_{max} or EM_{max}) values and the numerical difference, in nanometers, between these values is the Stokes Shift (Fig. 2.7). Each fluorochrome has a unique Stokes Shift and when choosing fluorochromes for flow cytometry, a large Stokes shift is desired because it means that the light emitted by the fluorochrome is more easily distinguished from the light needed to excite the fluorochrome [3].

As the photons of light are emitted from the excited fluorochrome, they are directed toward the collection optics, which consist of a set of PMTs that detect and amplify fluorescent light. The collection optics can be configured in either an octagon or trigon format (see the 488 octagon and 640 trigon in Fig. 2.3). The PMTs in both the octagon and trigon are coded with letters and the names of the fluorochromes specific for that PMT (ex. PMT A or AF750 is shown in the 640 trigon). Whereas the BD FACScan™ had only three PMTs, the five laser BD LSR Fortessa™ can have up to 18+ different PMTs for the detection of 20 parameters including FSC and SSC. Voltage settings applied to the PMTs determine how much of the fluorescent signal detected by a PMT is amplified. Voltages need to be high enough that weak signals can be distinguished from negative signals but low enough to prevent strong signals from being off-scale or extensively interfering with other fluoro-

Fig. 2.7 Stokes Shift. Schematic showing the $\text{Excitation}_{\text{MAX}}$ and $\text{Emission}_{\text{MAX}}$ for a representative fluorochrome. The Stokes Shift is the difference between the $\text{Excitation}_{\text{MAX}}$ and $\text{Emission}_{\text{MAX}}$



chromes with similar emission spectra. This is very similar to the way an amplifier works when connected to an electronic piano or keyboard.

The pianist has the ultimate control over the dynamics (louds and softs) of the notes they play based on how hard they strike the piano keys. The amplifier controls the balance between the loudest and softest tones such that the softest tones are not drowned out by the surrounding noise and the loudest tones do not over power the rest of the instruments in the band. It is important to note adjusting the voltage settings does not alter the intensity of the excitation lasers, only the sensitivity of the PMTs. The filters or more appropriately named, bandpass filters, are shown in a box directly below the PMTs, and have 2 sets of numbers on them to represent the wavelengths they will detect. Finally, below the bandpass filters are the long-pass mirrors that direct emission spectra to the appropriate PMTs. Both bandpass filters and long-pass mirrors will be discussed in more detail shortly in the example found in Fig. 2.5.

1.3 Electronics

The electronics system converts the emission spectra into digital signals to be analyzed by the computer using software such as BD FACSDiVa™ or other acquisition software on the market (Fig. 2.3). Early flow cytometers such as the BD FACSCalibur™ are analog, while modern flow cytometers such as the BD LSRFortessa™ are digital. With the BD LSRFortessa™, the voltages are digitized into one of 16,384 possible levels ten million times/s by Analog to Digital converters, or ADCs [2]. During this process of amplification and digitization, the voltage pulses are binned where they are assigned a height, width, and area. ADC resolution varies between instruments, but higher-bit ADCs have higher resolution. The ADC resolution is a measure of the bins the data can be assigned to. Signals are then transferred via Ethernet cables to the computer to display the data graphically. The electronics measure the area, height, and width parameters for FSC/SSC and

fluorescent spectra. Flow data plots are the result of these fluorescent signals being converted into meaningful data by the electronics of the Flow Cytometer (Fig. 2.5). Three main plots routinely used include the following:

- *Dot plots*: Two parameter plots (Fig. 2.8a) that show (1) cell size/granularity based on FSC and SSC (light scatter; left panel) or (2) protein expression on cells using antibodies conjugated to fluorochromes (fluorescence; right panel). The data is illustrated using individual cells or dots, and can be shown using monochromatic dot plots (e.g., black) or using pseudocolor dot plots. Pseudocolor

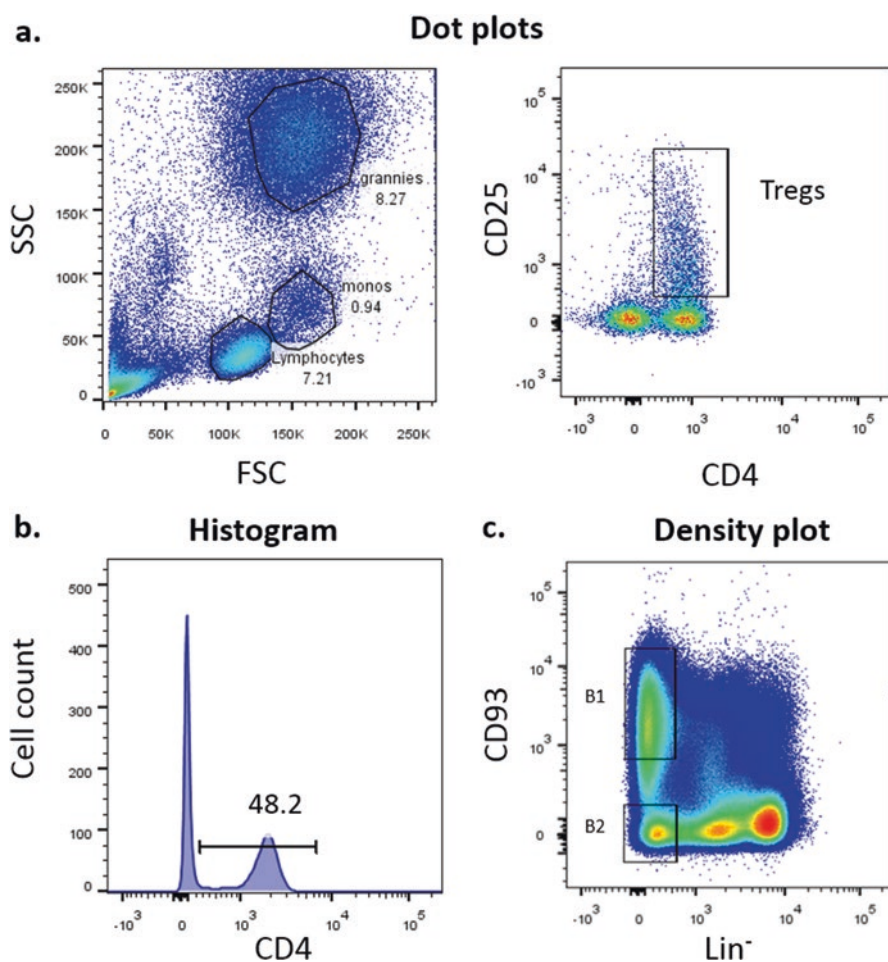


Fig. 2.8 Electronic conversion to Flow Cytometry Data Plots. (a, left panel) FSC/SSC plot of human whole blood. PBMCs were stained with CD4 and CD25 (a, right panel), CD4 (b), or a Lineage cocktail (Lin-) and CD93 (c). Cells were then acquired on a BD LSRFortessa™ and analyzed using FlowJo® software. The collected light emissions are displayed as dot plots, histograms, or density plots

dots plots are essentially heat maps that show the density of each cell population. Red indicates high expression, yellow to green is intermediate expression, and blue illustrates low expression of the proteins of interest on the cells.

- *Histograms*: One parameter plots that show cell number on the y-axis versus fluorescence or expression of your protein of interest on the x-axis (Fig. 2.8b).
- *Density plots*: Two parameter plots that show an accumulation and relative density of cells expressing the proteins of interest. The data is illustrated using contours (not individual cells as is the case with dot plots) where high/dense contouring indicates a high number of cells expressing the proteins of interest (Fig. 2.8c).

2 Putting It All Together

Figure 2.9 illustrates how the fluidics, optics, and electronics system work together during cell acquisition and analysis. There is a mixture of activated human T cells labeled with the fluorescent antibodies, CD4 FITC and CD127 A700. The fluidics system transports these cells in a single stream to the laser beams for interrogation in the flow cell. FSC and SSC are detected by the appropriate PMTs. The lasers excite FITC (CD4) and A700 (CD25) on the cells, and optical filters direct the emitted light from these fluorochromes to the appropriate PMTs. The Long-pass filters

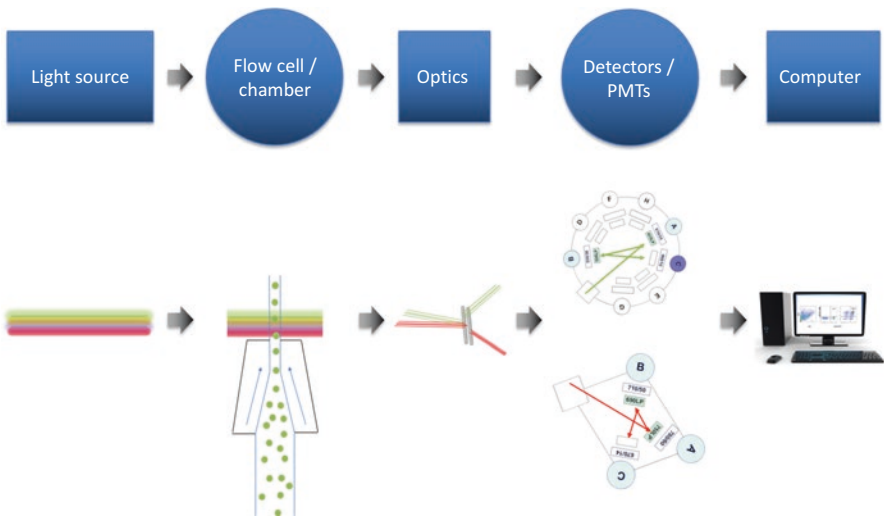


Fig. 2.9 Progression from antibody stained cells to flow cytometry data plots. Excitation lasers excite the fluorochrome-conjugated antibodies on cells within the flow cells. The emitted light is then focused by a series of mirrors and filters within the optics system and direct the light to the appropriate PMTs where the signal is amplified. An ADC converts the electronic pulses to channel numbers and these are displayed on the computer as flow cytometry data plots

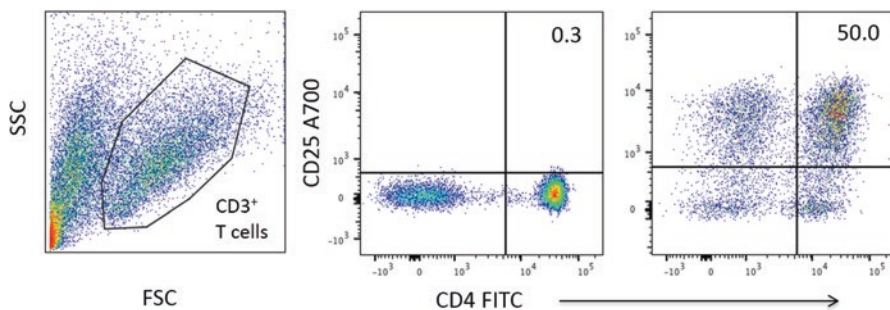


Fig. 2.10 Dot plot data readout of CD4⁺CD25⁺ human T cells. Human CD3⁺ T cells were purified from PBMCs, cultured in the presence of MxhCD3 (5 $\mu\text{g/mL}$)/GtxhCD28 (5 $\mu\text{g/mL}$) and cytokines (IL-2 (5 $\mu\text{g/mL}$), IL-7 (10 $\mu\text{g/mL}$), and IL-15 (10 $\mu\text{g/mL}$)) for 5 days, and then stained for CD4 FITC and CD25 A700. Cells were then acquired on a BD LSRFortessaTM and analyzed using FlowJo[®] software

divert emitted fluorescence spectra below the value listed to the next detector. For example, the A750 LP filter is labeled 750LP. In both the 488 octagon and 640 trigon, the laser path goes from the highest wavelength, or PMT A, to the lowest wavelength or PMT C. If you focus on the 640 trigon in Fig. 2.5, the laser path goes from the A750 PMT, fluorescent spectra below 750 nm then goes to the A700 PMT, and then anything below 690 nm goes to the APC PMT. These are also labeled alphabetically: A $\rightarrow$ B $\rightarrow$ to C. This happens simultaneously across all the octagons/trigons on the cytometer.

The bandpass filters restrict the range of emission spectra for each PMT to help reduce emission spillover, which will be discussed in Chaps. 3 and 5. The resulting data is then digitally converted by the electronics system. There is an ADC or analog to digital converter that converts electronic pulses to channel numbers [2] and these are displayed on the computer using the software such as FACSDivaTM (refer to Fig. 2.10 for flow data plots). This example describes a basic 2-parameter flow experiment. In Chap. 3, we will expand on this and lead you from experimental setup to acquisition and analysis using a 6-parameter flow experiment. We will also go into more detail on terminology associated with flow cytometry to familiarize you with terms used throughout this book.

3 Materials

3.1 Flow Antibodies

- Human CD4 (FAB3791; R&D Systems)
- Human CD25 A700 (FAB1020N; R&D Systems)
- Human CD93 (FAB23791; R&D Systems)

3.2 Cell Activation Reagents

- MxshCD3 (MAB100; R&D Systems)
- GtxhCD28 (AF-342-PB; R&D Systems)
- Recombinant Human IL-7 (207-IL; R&D Systems)
- Recombinant Human IL-2 (202-IL; R&D Systems)
- Recombinant Human IL-15 (247-ILB; R&D Systems)

3.3 Cell Purification Kits

- MagCelect™ Human CD3+ T cell isolation kit (MAGH101; R&D Systems)

3.4 Staining Buffers/Fc Block Reagents

- Flow Cytometry Staining Buffer (FC001; R&D Systems)
- Flow Cytometry Human Lyse Buffer (FC002; R&D Systems)
- Normal Human IgG (1-001-A; R&D Systems)
- Normal Mouse IgG

References

1. Golden JP, et al. Hydrodynamic focusing—a versatile tool. *Anal Bioanal Chem.* 2012;402:325–35.
2. Intro to flow cytometry: a learning guide. BD Biosciences. 2002; Manual Part Number: 11-11032-01.
3. Bhattacharya B, Singha S, Basu S. Fluorescent nanosensors: rapid tool for detection of food contaminants. *Nanobiosensors.* 2017:841–74.

Chapter 3

The Language of Flow Cytometry and Experimental Setup



Christine Goetz and Christopher Hammerbeck

Abbreviation

A405 = Alexa Fluor® 405

1 Flow Data Plots and Gating

1.1 Flow Data Plots

In Chap. 2, we briefly touched on the three main types of data plots that are used in flow cytometry: *histograms*, *dot plots*, and *density plots*. Histograms can be used to measure antibody fluorescence on the *x*-axis and cell number on the *y*-axis. Refer to Fig. 3.1a to see an example of CD8 expression using a histogram. Dot plots and density plots are 2-parameter plots that can be used to measure antibody fluorescence and/or light scatter (Forward Scatter or Side Scatter) on both the *x*- and *y*-axis. Listed below are some common parameters used together:

- Forward Scatter (FSC) vs. Side Scatter (SSC).
- Protein expression (fluorescence) vs. SSC (or FSC).
- Protein expression 1 (fluorescence) vs. Protein 2 expression (fluorescence).

FSC versus SSC is always employed when setting up an experiment to gate on the cell population of interest (this will be discussed in more detail later in this chapter). Combinations of these parameters can also be used where fluorescence is measured versus SSC in order to measure protein expression versus the size of the cell

Electronic Supplementary Material: The online version of this chapter (doi:[10.1007/978-3-319-98071-3_3](https://doi.org/10.1007/978-3-319-98071-3_3)) contains supplementary material, which is available to authorized users.

C. Goetz (✉) · C. Hammerbeck

Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA

e-mail: Christine.Goetz@bio-technie.com; Chris.Hammerbeck@bio-technie.com

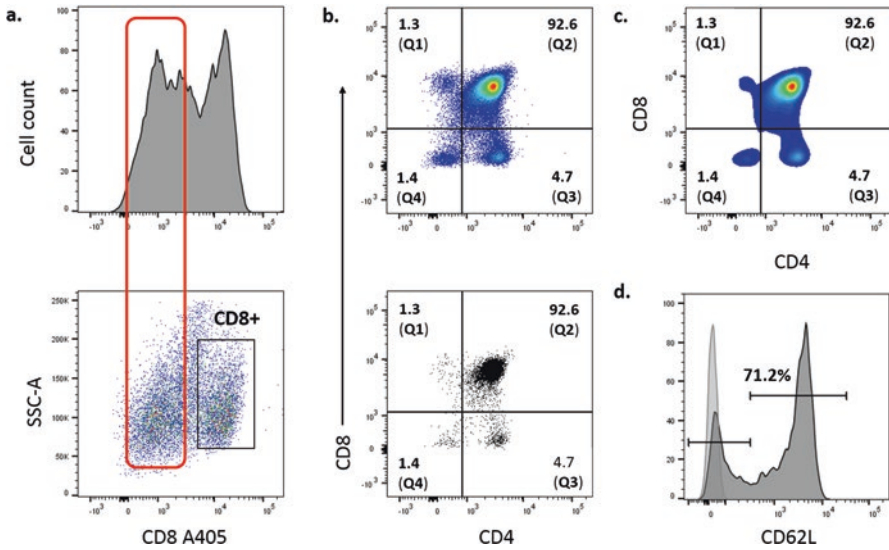


Fig. 3.1 Interpreting dot plots. Histograms, dot plots, and density plots are shown on various lymphocyte populations in human and mouse. Cells were harvested, stained with the antibodies indicated below, acquired on a BD LSRFortessa™ and analyzed using FlowJo® software. (a) hCD8 A405 expression is shown in human PBMCs using both a histogram (top panel) and dot plot using SSC-A (bottom panel). (b, c) Mouse thymocytes were stained for mCD4 and mCD8 and are shown using a pseudocolor dot plot (top panel, b), monochromatic dot plot (bottom panel, b), and density plot (c). (d) Human PBMCs were stained for hCD62L, and gates were set on the negative and positive populations

population of interest. This can be useful when it is necessary to use a dim fluorochrome. The difference can easily be seen when comparing CD8 A405 expression using a histogram (plotted against cell number; top panel) versus a dot plot where CD8 A405 is plotted against SSC (bottom panel). The separation is not clear using a histogram but is easier to distinguish using CD8 A405 vs. SSC (refer to the red box illustrating these differences in Fig. 3.1a). Protein expression can also be evaluated using a dot plot where Protein 1 is plotted versus Protein 2. Examination of CD4 and CD8 expression in mouse thymii illustrates this point nicely. Refer to Fig. 3.1b to see the four possible scenarios: CD4⁻CD8⁻ double negative cells, CD4⁺CD8⁻ cells, CD8⁺CD4⁻ cells, and CD4⁺CD8⁺ double positive cells. The CD4⁻CD8⁻ double negative cells (DN) are in the lower left quadrant (Q4) and neither protein is expressed. CD4⁺CD8⁻ cells are shown in Q3 and are said to be CD4 single positive. CD8⁺CD4⁻ cells are shown in Q1 and are said to be CD8 single positive. Finally, CD4⁺CD8⁺ or double positive (DP) cells are shown in Q2 and both proteins are expressed. Q1–4 stands for quadrants and this gating strategy will be discussed later.

Density plots (Fig. 3.1c) are similar to pseudocolor dot plots (Fig. 3.1b; top panel); both plot types display a 2-parameter representation of cell accumulation. Most researchers use pseudocolor dot plots when displaying their data as monochromatic dot plots do not give as accurate an indication of cell number (Fig. 3.1b, bot-

tom panel). Density plots use contours rather than dots (individual cells) to illustrate low or high numbers of cells or cell density (Fig. 3.1c). In general, these data plots measure the various fluorochromes on antibody-labeled cells that each flow cytometer is capable of detecting.

1.2 Gating

Gating is a commonly used phrase in flow cytometry that refers to selecting a particular region of data to analyze. Linear gates are used on histograms whereas polygons, rectangles, or circles can be used to gate on dot plots. There are four main types of gates: polygon gates (freeform gates; see FSC/SSC section), histogram gates, quadrants, and boxes. Each of these can be used to select cell populations of interest. For example, using the histogram gates in Fig. 3.1d, we can select CD62L⁺ (right gate) and CD62L⁻ populations (left gate). Cell populations identified with these gating strategies can then be further analyzed. Similarly, quadrant gates are frequently used to allocate protein expression into 4 regions: Q1, Q2, Q3, and Q4. The example of CD4 and CD8 expression in mouse thymii (Fig. 3.1b–c) is gated using quadrants. Similar to histogram gates, boxes can be used to gate on a particular cell population; in this example, CD8⁺ T cells are selected using a box gate (Fig. 3.1a, bottom panel). Gating is also hierarchical, meaning that gates build off the previous one. Outlined below is a typical gating hierarchy that would be used in mouse thymii stained with CD4 and CD8 (Fig. 3.1b, c). P1 or the live gate, is referred to as an upstream gate whereas the quadrant gates Q1–Q4 are downstream gates; they are downstream of P1. Therefore, quadrant gates 1–4 are said to be dependents of P1.

Typical gating hierarchy.

P1 (live gate)

- Q1 (CD8⁺CD4⁻)
- Q2 (CD8⁺CD4⁺)
- Q3 (CD8⁻CD4⁺)
- Q4 (CD8⁻CD4⁻)

2 Mean Fluorescence Intensity (MFI) and Biexponential vs. Logarithmic Scaling

Fluorescence and light scatter is measured using PMTs that convert the emission spectra into a data point on the computer that is represented graphically. Fluorescence is measured in log mode, where each flow plot is displayed logarithmically and ranges from 10⁰ (0), 10¹ (10), 10² (100), 10³ (1000), 10⁴ (10,000), and 10⁵ (100,000) in channel numbers. Each event that falls into a given channel corresponds to its signal intensity. Very low signal intensity is around 10⁰–10¹, whereas very high signal intensity would be around 10⁴–10⁵.

2.1 *Mean Fluorescence Intensity (MFI)*

Another way to describe the signal/fluorescence intensity of a conjugated antibody is to use the term **Mean Fluorescence Intensity** or MFI. It is calculated using the geometric, mean, or median index. An antibody with a high MFI has high signal intensity (we will discuss this more later). Fluorescence is defined as emission of light from fluorochromes on conjugated antibodies caused by excitation of lasers on the flow cytometer. When analyzing the expression pattern of a protein, both percentage and MFI are calculated. The MFI can be calculated using geometric mean, median, or mean index [1]. Mean is technically the arithmetic mean, but is simplified to mean in software databases such as BD FACSDIVA™ and FlowJo®.

The data in the statistics plot show the geometric mean, median, and mean MFI for hCD69 PE (Fig. 3.2a). Focus on the P2 gate where the MFI values differ using these three different parameters. The geometric mean MFI is more affected by outliers and skews toward them whether they are high or low. The geometric mean MFI can be useful for detecting proteins with weak expression [2]. The median MFI is set at 50%; where 50% of the cells are above or below the midpoint. Mean MFI is the average of the fluorescence intensities. It tends to skew to the right or toward higher MFIs. MFIs using geometric mean, median, and mean indexes do not vary much in value (all are around ~1900) for hCD4 A405 as the histogram is a tight peak (Fig. 3.2b). Therefore, the midpoint is similar for all these MFI parameters. In contrast, hCD69 PE has more of a broad distribution so the values vary greatly for geometric (8917), median (12,784), and mean (16,809) MFIs (Fig. 3.2c). The values calculated for geometric mean, median, and mean MFI were done using BD FACSDIVA™ software (Fig. 3.2a) versus FlowJo® software (Fig. 3.2c) so the values are a little different due to slight variances in the gating. However, the trends are the same: the geometric mean MFIs are the lowest, median MFIs are in the middle, and mean MFIs are the highest. In general, median MFI is probably the best parameter to describe signal intensity as its value lies between the geometric mean and mean; however, as mentioned above, there will be exceptions. Unless otherwise specified, MFI, for the remainder of this book, refers to the Median Fluorescence Intensity.

2.2 *Logarithmic vs. Biexponential Scaling*

Fluorescence MFIs are shown in log mode ($0-10^5$). There are two scaling options available: logarithmic or biexponential scaling. Biexponential scaling allows visualization of values below zero. In contrast, logarithmic scaling compresses the channels of visual space as the scale increases. There are a few reasons for this. First, with compensation error or spreading, negative fluorescence is observed. Second, there is fluorescent baseline subtraction error during acquisition. With all these combined, the first decade becomes smashed or piled up [3, 4]. See Fig. 3.3 to see

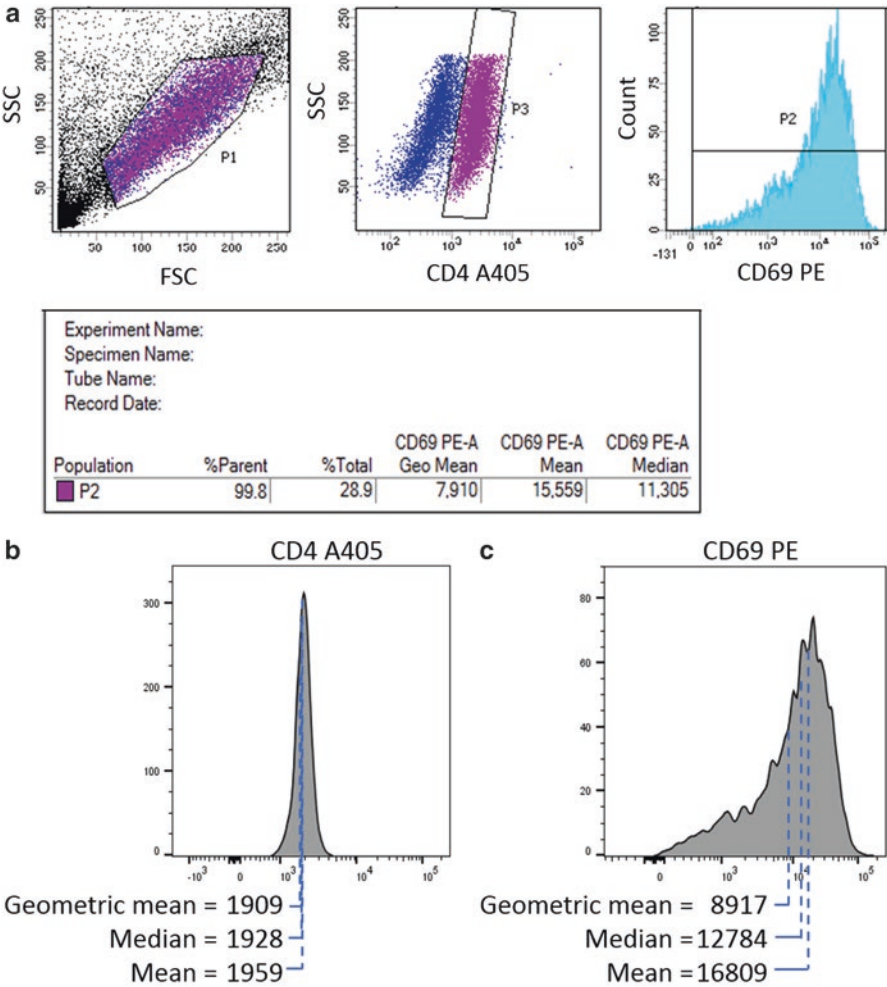


Fig. 3.2 Geometric mean, Median, and Mean MFIs. Human PBMCs were activated for 2 days with MxhCD3 (2.5 µg/mL) and GtxhCD28 (2 µg/mL) to induce T cell activation. Cells were harvested and stained with hCD4 A405 and hCD69 PE. Cells were then acquired on a BD LSRFortessa™ and analyzed using FlowJo® software. (a) Cells were gated on blasting live lymphocytes and CD4⁺ T cells. CD4⁺CD69⁺ T cells are shown in the histogram. Statistics table of geometric mean, median, and mean MFI on the Fortessa for hCD69 PE. (b) Geometric mean, median, and mean MFI values for hCD4 A405. (c) Geometric mean, median, and mean MFI values for hCD69 PE

an example of hCD4 versus hCD8 expression using logarithmic scaling (top panel) versus biexponential scaling (bottom panel). There is more visual separation of these mutually exclusive T cell subsets with biexponential scaling. Biexponential scaling compresses the data in the lower range so that populations with low fluorescence can be seen.

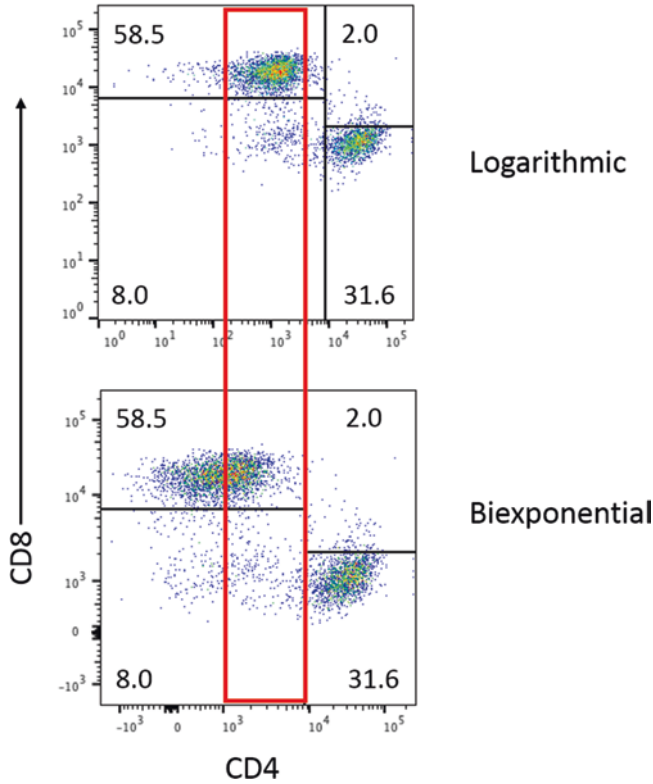


Fig. 3.3 Logarithmic versus Biexponential scaling. Human PBMCs were stained for hCD4 and hCD8, acquired on a BD LSRFortessa™ and analyzed using FlowJo® software. Logarithmic scaling is shown in the top panel and biexponential scaling is shown in the bottom panel

3 Forward Scatter (FSC)/Side Scatter (SSC) and Doublet Exclusion

3.1 FSC/SSC

Flow cytometry makes use of cell size and granularity to help define characteristics of a cell population. As discussed in the previous chapter, cell size is measured by FSC, and the complexity of a cell is measured by SSC. Specifically, FSC measures light scattered forward and around the cell. In addition to measuring cell size, FSC also takes into account nuclear to cytoplasmic ratio, membrane topography, and other cellular characteristics. SSC measures light scattered perpendicular to the laser beam and particulates inside the cell. SSC measures cell complexity and granularity; granularity refers to how many small particles or granules are present within the cell.

As mentioned above, FSC and SSC measure light scatter, not fluorescence, and are used to gate on your cell population of interest. These parameters are both run

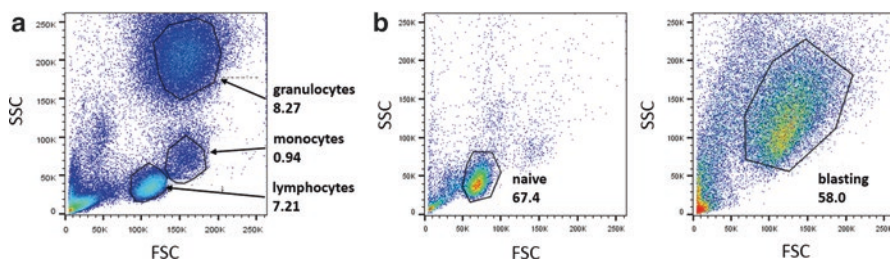


Fig. 3.4 FSC/SSC of Whole blood and Naive versus Blasting T cells. **(a)** The FSC/SSC profile is shown for human whole blood. The three main cell populations include the lymphocytes, monocytes, and granulocytes. **(b)** FSC/SSC profiles were acquired on a BD LSRFortessa™ for either naive CD3⁺ or Th1 CD3⁺ human T cells. For expansion of hTh1 cells, CD3⁺ T cells were cultured in the presence of Th1 promoting stimuli (MxhCD3 (2.5 µg/mL), GtxhCD28 (2 µg/mL), and rhIL-12 (10 ng/mL) for 5 days. The FSC/SSC profiles are shown for both the naive and blasting CD3⁺ T cell populations. Data was analyzed using FlowJo® software

in linear mode ranging from 0 to 250,000. Large cells have high values for FSC and SSC, and are located in the upper right portion of the FSC/SSC plot (see Fig. 3.4a). In contrast, small cells such as red blood cells have low FSC values; however, very few are present in this FSC/SSC dot plot as they have been lysed. The FSC/SSC profile of human whole blood illustrates this concept nicely (refer to Fig. 3.4a). Polygon gates have been drawn around each of the populations in whole blood: lymphocytes, monocytes, and granulocytes. Polygon/live cell gates should be drawn generously to include the entire cell population for each; restrictive gating can be misleading for downstream protein expression. Lymphocytes are small cells and not very granular; therefore, their FSC and SSC values are low. Monocytes are bigger cells and are more granular so their FSC and SSC values are higher; the cell population is shifted further to the right on the x-axis and up a bit on the y-axis. Finally, as their name implies, granulocytes are granular and are also large so they have high FSC and SSC values. In addition, blasting cells have high FSC as they are large; this is commonly seen during cell activation and differentiation. Refer to Fig. 3.4b where the left dot plot shows the FSC/SSC profile of naive hCD3⁺ T cells and the right dot plot shows the FSC/SSC profile of blasting hCD3⁺ T cells that have been polarized with Th1 stimuli for 5 days. The naive CD3⁺ T cells have low FSC/SSC relative to the blasting CD3⁺ T cells. When drawing gates on naive vs. blasting cells, it is important that you include all relevant cells in the live gate. For example, many cells would be missed if the naive gate (small gate) was applied to the blasting cells.

3.2 Doublet Exclusion

Use of FSC/SSC plots not only allows gating on the cells of interest, but also aids in excluding cellular debris and dead cells. Dead cells are typically very small, and have low FSC and SSC. For example, in Fig. 3.5 (top left FSC/SSC plot), it is

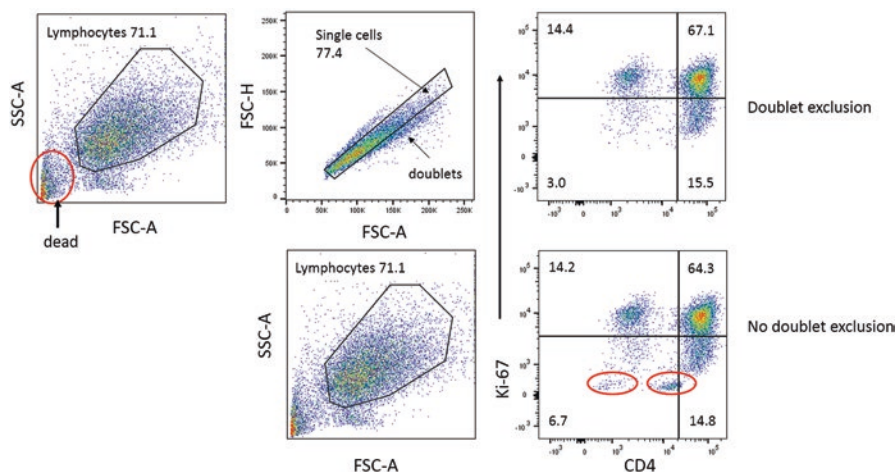


Fig. 3.5 Doublet exclusion. Human CD3⁺ T cells were purified and then cultured for 5 days in the presence of Th1 promoting stimuli (MxhCD3 (2.5 $\mu\text{g/mL}$), GtxhCD28 (2 $\mu\text{g/mL}$), and rhIL-12 (10 ng/mL)). After harvesting the cells, cells were stained for hCD4 and Ki-67 expression, run on a BD LSRFortessaTM and analyzed using FlowJo[®] software. Doublet exclusion is applied in the top set of panels. The bottom set of panels show CD4 and Ki-67 expression solely off the live lymphocyte gate

obvious there is a small population of dead cells/debris in the lower left part of the dot plot circled in red. The population that has a black polygon drawn around it is the live population of interest; however, there are still some dead cells in our live gate. Doublets are typically dead cells that are stuck together; they have a high FSC value due to aggregation. However, doublets can also be live cells that have aggregated due to upregulation of integrins and adhesion molecules. Doublets are evident in the lower right dot plot (Fig. 3.5) showing CD4 versus Ki-67 (a nuclear protein associated with proliferation) and are highlighted with red circles.

The diagonal gate that extends from the bottom left corner to the top right corner is the doublet-free or single-cell population (see the gate drawn around the cells in Fig. 3.5, middle top dot plot). The single-cell gate has been applied to the data in the top right dot plot, and as a result, the data for CD4 versus Ki-67 is clean and free of debris. This is especially important to consider if the cells have been activated with potent stimuli (such as PMA/Ionomycin) or polarized for an extensive period of time to allow for differentiation (the example in Fig. 3.5 shows hTh1 differentiation for 5 days). Cell death and aggregation is very common in these instances, and inclusion of doublets can confound analysis of surface and intracellular protein expression. Dead exclusion dyes are also available to help discriminate between live and dead cells and will be discussed in Chap. 7.

4 CS&T and Basic Experimental Setup

4.1 CS&T

Prior to running an experiment on a flow cytometer, it is important to run quality checks on a daily basis to ensure the cytometer is functioning properly. Cytometer **Setup and Tracking** (CS&T) beads allow end users to do these quality checks and ascertain that the lasers are in alignment (lasers drift over time and have to be adjusted) and that the fluidics system is working properly. Preventative maintenance is done annually to keep these problems in check. For more details on how to perform CS&T, consult your flow cytometer's website and technical support. For the BD LSRFortessa™ (Fortessa), refer to the following weblink [5].

4.2 Basic Experimental Setup

4.2.1 Experimental/Flow Panel Design

Panel design and experimental acquisition and analysis will be described in this section. Consider a 6-color experiment using induced Tregs (iTregs) from human PBMCs. PBMCs have been stimulated in vitro with IL-2 and TGF- β for 2 days to induce Treg expansion. CD4⁺ T cells are the primary T cell subset that produces Tregs, so this is one of our costains. Costains are antibodies that are used in conjunction with the primary antibody of interest to define the phenotype and/or functional characteristics of a cell population. CD39 and CD25 are surface markers expressed on Tregs whereas HLA-DR is negative on Tregs. Both Helios and FoxP3 are transcription factors expressed on Tregs. Here is a list of the 6-color flow panel:

- Lineage marker: CD4 A405
- Surface markers: CD39 A488, CD25 A700, HLA-DR PerCP
- Transcription factors: Helios PE, FoxP3 A647

4.2.2 Surface and Intracellular Staining on iTregs

Listed below is a brief description of how we stain the cells for these proteins (refer to Chap. 9 for a detailed protocol):

1. Isolate cultured iTregs. Wash in flow cytometry staining buffer twice, and block with human and mouse IgG.
2. Surface stain for CD4, CD39, CD25, and HLA-DR. Wash twice after staining with 1× PBS.
3. Fix and permeabilize cells with the FoxP3 buffer set.
4. Carry out intracellular staining with Helios and FoxP3.
5. Wash cells with 1× permeabilization buffer and run on the Fortessa.

4.2.3 Experimental Setup on the Fortessa

Here are the basic steps to follow for experimental setup on the Fortessa:

1. Open the Diva™ software on the Fortessa, then create and name a new experiment.
2. Create a specimen from the dropdown menu, name it, and create the isotype control and antibody tubes (and label each sample tube). These will be used for sample acquisition.
3. Select which parameters are being used and delete those that are not needed. For the fluorescent parameters, be sure log mode is selected along with area for A405, A488, PE, PerCP, A647, and A700. Delete the unused fluorescent parameters as this will increase the byte size of the flow file.
4. Create dot plots for FSC/SSC and each of the fluorochrome combinations.
5. Acquire sample and draw a polygon gate (P1) around the live cells. Adjust the voltages for FSC and SSC as needed to make sure the live cell population is on scale. Signals are amplified or decreased by increasing or decreasing the voltage, which changes the electrical current for the PMTs. Create the population hierarchy by right clicking on the FSC/SSC plot; this allows visualization of the gating hierarchy.
6. Select all the dot plots with fluorochromes and right click to apply biexponential scaling and the P1 gate to each plot. After this is done, acquire a small number of events (cells) to assess if the voltages are set correctly for each fluorochrome. Adjust PMT voltages as needed so that the data is on scale. Sample cells are being used to set the PMT voltages, not compensation beads. (Compensation beads are used only for compensation.)
7. Some fluorochrome combinations (Fig. 3.6a, top four dot plots) will require compensation as there is a diagonal of cells (or “winging” of cells) extending into the upper right quadrant. (Refer to Chap. 5 for more details on compensation). The bottom two dot plots require minimal/no compensation (Fig. 3.6b).

4.2.4 Sample Acquisition and Analysis

8. Assume that compensation has been carried out; acquire the isotype control and antibody samples (see Fig. 3.7). Since iTregs are a small population in peripheral blood, the collection gate should be set on CD4⁺FoxP3⁺ cells (circled in red; Fig. 3.7b) to ensure enough events are collected (1000 is sufficient). The collection gate refers to the gate defining the final target population of interest and total number of cells to be collected from within that defined population (iTregs in this case). This is different from the total event gate, which is the total number of cells collected (including those outside the collection gate) during acquisition.
9. The resulting Flow Cytometry Standard (FCS) files (select FCS 2.0) can be exported into an analysis program such as FlowJo® and analyzed. In Fig. 3.8, the definitive iTreg plots are outlined: CD4/FoxP3, CD25/FoxP3, CD25/Helios,

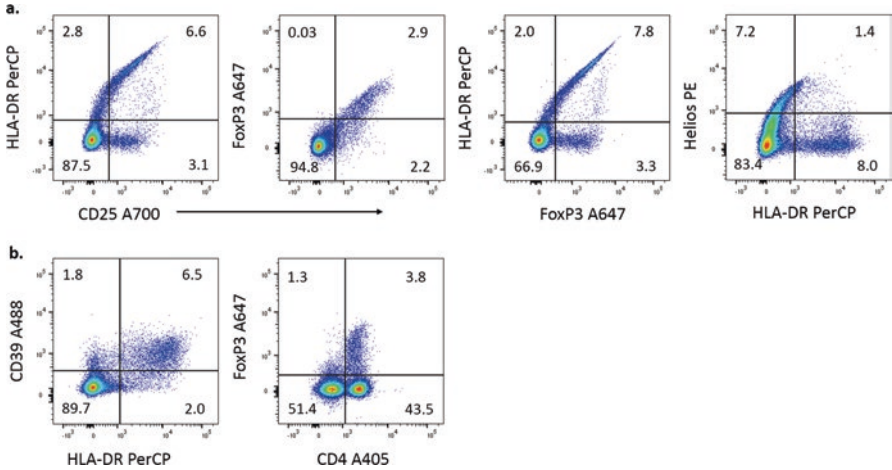


Fig. 3.6 Uncompensated samples for iTreg acquisition. **(a, b)** Human PBMCs were placed into culture for 2 days with IL-2 (20 ng/mL) and TGF- β (10 ng/mL) to induce Treg expansion (iTregs). Cells were harvested, and stained with hHLA-DR PerCP, hCD25 A700, hFoxP3 A647, hCD4 A405, hCD39 A488, and hHelios PE. Cells were then acquired on a BD LSRFortessa™ and analyzed using FlowJo® software

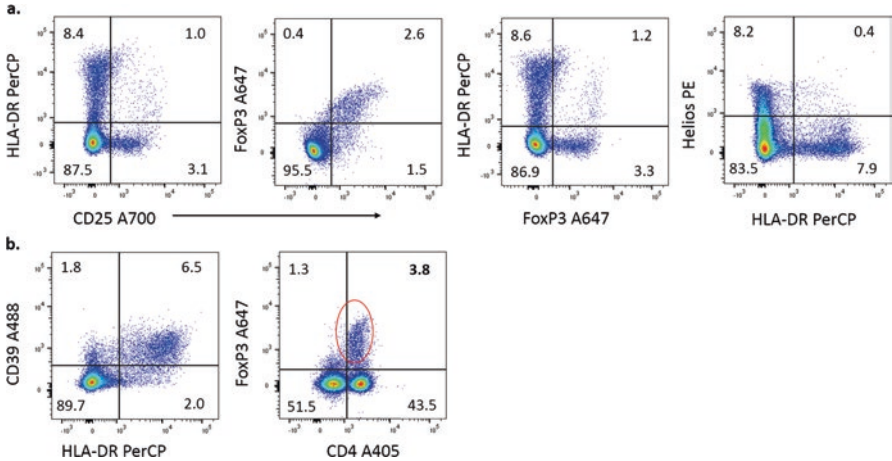


Fig. 3.7 Compensated samples for iTreg acquisition. **(a, b)** Human PBMCs were placed into culture for 2 days with IL-2 (20 ng/mL) and TGF- β (10 ng/mL) to induce Treg expansion (iTregs). Cells were harvested, and stained with hHLA-DR PerCP, hCD25 A700, hFoxP3 A647, hCD4 A405, hCD39 A488, and hHelios PE. Cells were then acquired on a BD LSRFortessa™, compensated using BD FACSDIVA™ software, and analyzed using FlowJo® software

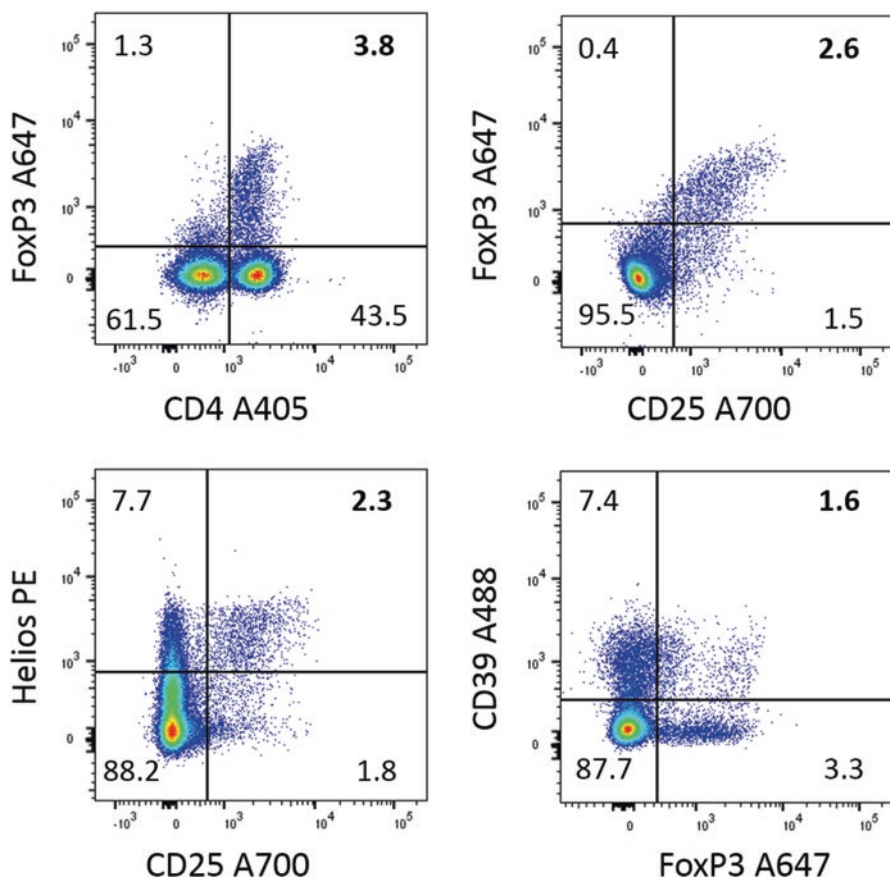


Fig. 3.8 Analysis of iTregs in FlowJo® post acquisition on the BD LSRFortessa™. After acquisition on the BD LSRFortessa™, data files were analyzed in FlowJo®. iTregs were identified using the following dot plots: CD4/FoxP3, CD25/FoxP3, CD25/Helios, and FoxP3/CD39

and FoxP3/CD39. In each of these plots, the percentage of iTregs is low or 1.6–3.75% depending on the antibody used for gating. However, the iTreg populations are clear using these markers and support the percentages cited in the literature.

For a detailed video on how this experiment was carried out on the Fortessa, please refer to the Supplemental Online Video.

5 Basic Concepts of Compensation

In the experiment just outlined, compensation was necessary with some of the fluorochrome combinations. Compensation is also referred to as fluorescence spillover and is required when performing multicolor flow cytometry. Compensation is

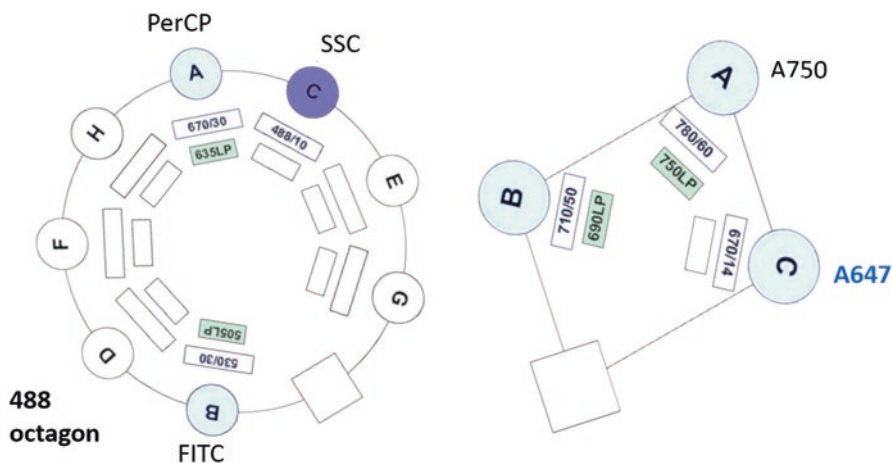


Fig. 3.9 Schematic of the 488 octagon and 640 trigon on the BD LSRFortessa™. The various PMTs are represented on the BD LSRFortessa™ for the A488 octagon and 640 trigon (BD LSRFortessa™ cytometer configuration window). The bandpass filters are shown in rectangular boxes under the alphabetical letter representing each PMT. Longpass mirrors are shown directly below the bandpass filters

simply defined as subtracting emission spectral overlap as a percent between multiple fluorochromes; this means that some signal will be lost across each fluorochrome that is compensated. Compensation is necessary to allow for accurate measurement of each fluorochrome as uncompensated samples may give false data as we'll show in Chap. 5. Compensation beads are commonly used to assist in compensation that can be done either manually or using the Autocompensation feature using FACSDiva®. Compensation beads are species-specific beads and are stained with each antibody in your panel. These are carried out as single stains. A six-color panel will therefore have six separate compensation tubes containing beads stained with each antibody-conjugated fluorochrome and a seventh tube containing unstained beads. This will be discussed in more detail in Chap. 5.

Given that compensation is necessary in multicolor flow panels, bandpass filters help minimize this emission spillover. This is accomplished by bandpass filters restricting the range of emission spectra for each PMT. To visualize this concept, locate the A647 PMT highlighted in blue in Fig. 3.9. The A647 bandpass filter is 670/14; to calculate the emission range, split 14 so that 7 is subtracted from 670 and added to 670. The emission range then becomes 663–677. This gives a narrow emission range to minimize spillover into other fluorochromes that are close in wavelength such as A700 and A750.

Spectral overlap also gives an indication of how much compensation will be required between fluorochromes. Spectrum Viewers are available on the Novus Biologicals website (www.novusbio.com) and are quite useful in estimating the degree of spectral overlap between fluorochromes. The graphs in Fig. 3.10 show the various wavelengths across the light spectrum from low to high where the low end

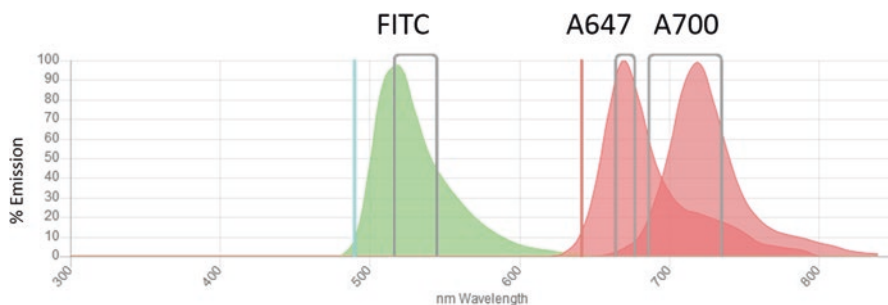


Fig. 3.10 Spectral Overlap of FITC, A647, and A700. Spectral overlap is shown using the spectrum viewer on the Novus website. The 488 laser line is shown in blue and the 640 laser line is shown in red. The emission spectra for each PMT are represented by a histogram. FITC is in green, and the red histograms represent either A647 or A700. The emission range is indicated by the bandpass filters in gray

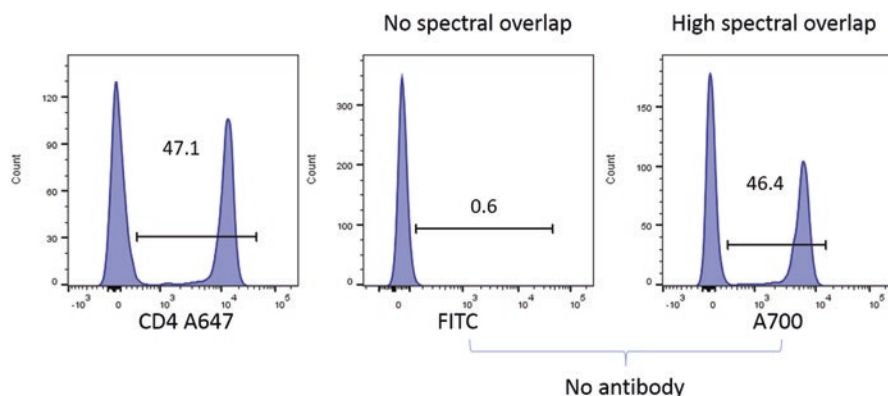


Fig. 3.11 Example of Spectral Overlap with hCD4 A647. Human whole blood was stained with hCD4 A647, acquired on a BD LSRFortessa™ and analyzed using FlowJo® software. Data was acquired on all PMTs to show the degree of spectral overlap between the various PMTs. The FITC, A647, and A700 PMTs are shown in this example

represents the violet laser fluorochromes and the high end represents fluorochromes off the red laser. The laser lines are included in this example; blue represents the 488 laser and red represents the 640 laser. A647 and A700 are on the same laser so there is considerable overlap (see how the red histograms merge into each other). Notice that A647 “bleeds” significantly into the A700 bandpass filter and that A700 doesn’t bleed nearly as much. Therefore, the compensation with A647 into A700 will be substantial. However, FITC is not on the same laser, so there is no compensation required between FITC and either A647 or A700 as there is no spectral overlap. Bandpass filter ranges are available on spectrum viewers and are indicated by the gray boxes. These are used to calculate compensation on the flow cytometer. Spectrum viewers will also be discussed in more detail in Chap. 4.

To illustrate the concept of spectral overlap, refer to Fig. 3.11 where human PBMCs have been stained with *only* hCD4 A647. In the far-left histogram, the

CD4⁻ and CD4⁺ cell populations are present as expected. The FITC and A700 histogram plots have been included during cell acquisition, but only the hCD4 A647 antibody has been used to stain the PBMCs. There is no spillover of CD4 A647 in the FITC channel (middle panel). In contrast, there is substantial spillover into the A700 channel (far right panel). This is because A700 and A647 are close in wavelength; the A700 channel picks up the A647 antibody. This example illustrates why it is critical to carry out compensation. Compensation can be a difficult concept to understand and master. To simplify this concept, we have included some practical examples, along with a video in Chap. 5, showing how to carry out compensation on the Fortessa.

6 Materials

6.1 *Flow Antibodies*

- Human/Mouse FoxP3 (IC8214; R&D Systems)
- Mouse CD4 (FAB554; R&D Systems)
- Mouse CD8 (FAB116; R&D Systems)
- Human CD4 (FAB3791; R&D Systems)
- Human CD4 A405 (FAB3791V; R&D Systems)
- Human CD4 A647 (FAB3791R; R&D Systems)
- Human CD8 (FAB1509; R&D Systems)
- Human CD8 A405 (FAB1509V; R&D Systems)
- Human CD25 A700 (FAB1020N; R&D Systems)
- Human CD39 A488 (FAB4397G; R&D Systems)
- Human CD62L (FAB9787; R&D Systems)
- Human CD69 PE (FAB23591P; R&D Systems)
- Human HLA-DR PerCP (FAB4869C; R&D Systems)
- Human Helios PE (IC73092P; R&D Systems)
- Human Ki-67 (FAB7617; R&D Systems)

6.2 *Cell Activation Reagents*

- MxhCD3 (MAB100; R&D Systems)
- GtxhCD28 (AF-342-PB; R&D Systems)
- Recombinant Human IL-12 (219-IL; R&D Systems)
- Recombinant Human IL-2 (202-IL; R&D Systems)
- Recombinant TGF-beta (100-B; R&D Systems)

6.3 *Cell Purification Kits*

- MagCelect™ Human CD3+ T cell isolation kit (MAGH101; R&D Systems)

6.4 *Staining Buffers/Fc Block Reagents*

- Flow Cytometry Fixation buffer (FC004; R&D Systems)
- FlowX FoxP3/Transcription Factor Fixation & Perm Buffer Kit (FC012; R&D Systems)
- Flow Cytometry Staining Buffer (FC001; R&D Systems)
- Flow Cytometry Human Lyse Buffer (FC002; R&D Systems)
- Normal Human IgG (1-001-A; R&D Systems)
- Normal Mouse IgG
- Flow Cytometry Permeabilization/Wash Buffer (FC005; R&D Systems)

References

1. Mean, Median, Mode. FlowJo templates part 2. 2007. http://flowjo.typepad.com/the_daily_dongle/2007/10/mean-median-mod.html.
2. Wu Y-J, et al. Using the geometric mean fluorescence intensity index method to measure ZAP-70 expression in patients with chronic lymphocytic leukemia. *OncoTargets Ther.* 2016; 9:797–805.
3. McNeill LK, et al. A harmonized approach to intracellular cytokine staining gating: Results from an international multic consortia proficiency panel conducted by the Cancer Immunotherapy Consortium (CIC/CRI). *Cytometry A.* 2013;83(8):728–38.
4. Parks DR, Roederer M, Moore WA. A new “Logicle” display method avoids deceptive effects of logarithmic scaling for low signals and compensated data. *Cytometry A.* 2006;69A:541–51.
5. <http://www.bdbiosciences.com/us/instruments/clinical/cell-analyzers/bd-facsanto/bd-facs-canto-10-color-set-up-reagents/csampt-research-beads-use-with-bd-facsdiva-software-v7-or-later/p/655050>

Chapter 4

Fluorochrome Choices for Flow Cytometry



Christine Goetz and Christopher Hammerbeck

Abbreviation

A647 = Alexa Fluor® 647

1 Fluorochromes and Their Pros and Cons

There are multiple resources available for choosing fluorochromes, and two that we highly recommend are the spectrum viewers available through Novus Biologicals and BD Biosciences. Table 4.1 lists some of the common fluorochromes available on the market today (Novus Biologicals). These range on the light spectrum from low wavelengths such as UV (350 nm), up to infrared (640 nm). As mentioned in Chap. 2, each fluorochrome is unique as each one emits light at a specific wavelength. This occurs because each of the fluorochromes exhibits its own Stokes shift (refer to Chap. 2; [1]).

The list of fluorochromes available via BD Biosciences is more comprehensive and includes more of the UltraViolet dyes, Brilliant Violet dyes, and tandems. The Alexa Fluors were invented by Molecular Probes and are small molecule dyes that are very bright and photostable. Fluorochrome photostability means that fluorochromes remain intact when exposed to light and are not prone to photobleaching (or falling apart; see below). DyLight dyes were produced by Dyomics and Thermo Fisher Scientific. DyLight Fluors are similar to the Alexa Fluors as they are bright, photostable, and pH-stable. The Janelia Fluor® dyes were invented by Luke Lavis (available through Tocris Bioscience) and are up to 50× brighter than traditional fluorochromes due to an azetidine ring included in the structure. While they are similar to Alexa Fluors in their photostability, they differ in that they are cell permeable [2]. With these newer fluorescent dyes, such as the DyLights, Dyomics, Janelia

C. Goetz (✉) · C. Hammerbeck

Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA
e-mail: Christine.Goetz@bio-technie.com; Chris.Hammerbeck@bio-technie.com

© Springer Nature Switzerland AG 2018

C. Goetz et al., *Flow Cytometry Basics for the Non-Expert*, Techniques in Life Science and Biomedicine for the Non-Expert,
https://doi.org/10.1007/978-3-319-98071-3_4

Table 4.1 Fluorochrome choices (novusbio.com)

350 Laser	405 Laser	488 Laser	561 Laser	640 Laser
DyLight® 350	Alexa ®Fluor 405	7C	DyLight® 594	Alexa Fluor® 647
AMCA	DyLight® 405	Alexa Fluor® 488	Dyomics 590	Alexa Fluor® 700
	DyLight® 405LS	Alexa Fluor® 546	DyLight® 549	APC
	Pacific blue™	Alexa Fluor® 555	DyLight® 550	APC-Cy5.5
		ATTO 488	Dyomics 547	APC-Cy7
		Cy2	Cy3	Cy5
		DyLight® 488	Cy3.5	Cy5.5
		FITC/fluorescein	Janelia Fluor® 549	Cy7
		HiLyte 488	Janelia Fluor® 585	DyLight® 633
		TRITC	PE	DyLight® 649
			PE-Cy5	DyLight® 650
			PE-Cy5.5	DyLight® 680
			PE-Cy7	DyLight® 755
			PE-Dyomics 647	DyLight® 800
			PE-Texas red	Janelia Fluor® 635
				Janelia Fluor® 646

Fluors, and Alexa Fluors, the names are based on their peak emission spectra. For example, with Alexa Fluor® 647 or A647, the peak emission is at 647 nm off the red laser.

FITC (Fluorescein isothiocyanate), APC (Allophycocyanin), and PE (Phycoerythrin) were some of the first dyes available for purchase. FITC is smaller in size than PE and APC (389 Da compared to 250 kDa and 105 kDa, respectively) and is moderately bright. PE and APC are very bright but are also large and can interfere with conjugation to certain antibodies due to issues with steric hindrance [3]. Tandem dyes to both PE and APC have been made to increase the number of fluorochromes available for researchers. Tandem fluorochromes that are coupled to APC or PE are typically Cyanine 5 (Cy5), Cyanine 5.5 (Cy5.5), or Cyanine 7 (Cy7). Tandem dyes involve the covalent coupling of two fluorochromes where one serves as an acceptor molecule and the other serves as a donor to create a new fluorochrome. Tandem dyes have the excitation properties of the donor and emission properties of the acceptor. An example is PE-Cy7 where PE is the donor and Cy7 is the acceptor molecule. Tandems are prone to photobleaching (meaning they are prone to dissociation, fading, or degradation with prolonged light exposure) and can also produce spillover in the channel of the parental dye. For example, an antibody conjugated to PE-Cy7 can spillover into the PE channel [4].

The BD Horizon Brilliant UV dyes and Violet dyes offer many bright fluorochrome options on the UV and Violet laser (see Table 4.2). This is advantageous as many high-density markers, cell proliferation dyes, and dead cell exclusion dyes

Table 4.2 Common UV and violet fluorochrome choices

UV (355 Laser)	Violet (405 Laser)
BUV395	BV421
BUV496	V450
BUV661	BV510
BUV737	V500
BUV805	BV605
	BV650
	BV711
	BV786

can be used on these two lasers without any compromise in signal intensity. Researchers typically reserve the 488, 561, and 640 lasers for their low-density markers and also due to limitations of antibody availability in other fluorochrome conjugations.

2 Spectrum Viewers and How to Use Them

When choosing fluorochromes for your experimental panel, it is useful to use the spectrum viewers available online to figure out what degree of spectral overlap to expect. We briefly described this in the previous chapter and will go over how to use it in more detail here. Illustrated below is a step-by-step outline of how to select fluorochromes and their respective bandpass filters on the spectrum viewer accessed via the Novus website (refer to Fig. 4.1).

1. Select each fluorochrome in your panel from the dropdown window.
2. Select the laser lines from the drop-down menu so you can visualize where each of the fluorochromes is falling with respect to the lasers they are on.
3. Select the bandpass filter for each of your fluorochromes. This will give you a sense of what degree of compensation there will be between each of the fluorochromes with high spectral overlap. Remember that compensation is correction for emission spillover and the bandpass filters are designed to minimize this.
4. Review the degree of spectral overlap and emission spillover. Adjust your panel as needed for pairing high-density markers with dim fluorochromes or those with high compensation.

3 Fluorochrome Combinations to Use/Avoid Together

One key point to keep in mind is that the spectrum viewers do NOT account for fluorochromes excited by different lasers so spectral overlap can appear high in some cases when it isn't an issue. For example, A594 and A647 are excited by separate



Fig. 4.1 How to use a spectrum viewer. Illustrated below is a step-by-step guide on how to use the spectrum viewer on the Novus website. (1) First, select the fluorochromes of interest. (2) Second, select the desired laser lines. (3) Third, select and enter the bandpass filter ranges for each fluorochrome. (4) Fourth, review the spectral overlap for all the fluorochromes in your panel

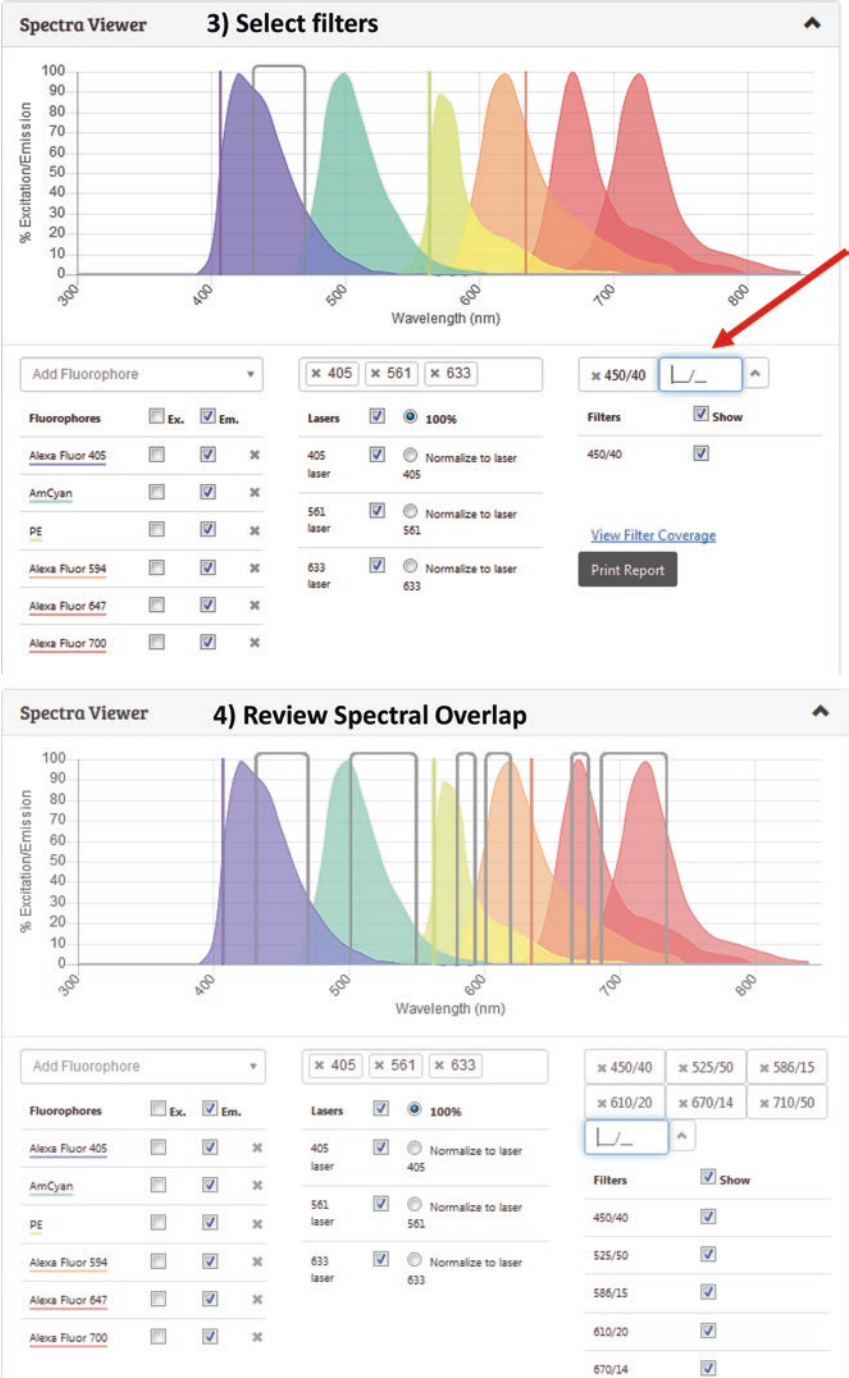


Fig. 4.1 (continued)

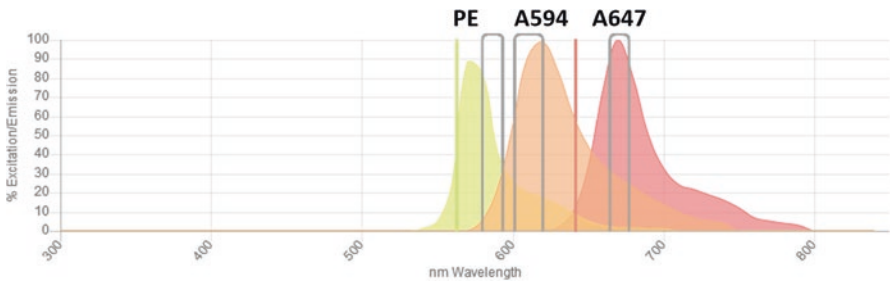


Fig. 4.2 Spectral overlap between PE, A594, and A647. Using the spectrum viewer on the Novus website, emission spectra was plotted for PE, A594, and A647. Laser lines (561 and 640) and bandpass filter ranges were also selected. Each histogram depicts the fluorescence emission spectra for either the PE (yellow), A594 (orange), or A647 (red) PMT

Table 4.3 Fluorochrome choices on the BD FACSCalibur™

Dye	Laser	Bandpass filter	Brightness	Dyes with spectral overlap
PE-Cy7	488	780/60	Bright	PE, PerCP, APC
PerCP	488	695/40	Dim	PE, FITC, APC, PE-Cy7
PerCP-Cy5.5	488	695/40	Bright	PE-Cy7, PerCP, APC
A488/FITC	488	530/30	Bright	PE, PerCP
PE	488	585/42	Very bright	FITC, PerCP, PE-Cy7
APC/A647	635	661/16	Very bright	PerCP, PerCP-Cy5.5, PE-Cy7

lasers (561 and 640 lasers, respectively) so the degree of spectral overlap is very low. However, when you graph these two fluorochromes together using a spectrum viewer (refer to Fig. 4.2), their spectral overlap appears to be high. As expected, the spectral overlap between PE (yellow histogram) and A594 (orange histogram) is high. Therefore, you must know your flow cytometer’s PMT setup well before designing a panel. Now we will go over fluorochromes available on two of the most commonly used flow cytometers used in research labs: the BD FACSCalibur™ (Calibur) and BD LSRFortessa™ (Fortessa).

On the Calibur, the left column shows commonly used fluorochromes available on each laser (refer to Table 4.3). Some are bright such as PE and APC, whereas others are dim such as PerCP. In addition, each of these fluorochromes has some degree of spectral overlap with others close in wavelength. All these factors should be considered when choosing which fluorochromes to include in your panel, and which ones should be paired with high- or low-density proteins as we will discuss later.

The same points we made with the Calibur apply to the fluorochromes available on the Fortessa (Table 4.4). More are available as there are four lasers, and while most are bright, a few are dim such as A405 on the 405 laser and A750 on 640 laser. Again, compensation will be an issue with many of these fluorochromes, so panels need to be chosen with care.

Table 4.4 Fluorochrome choices on the BD LSRFortessa™

Dye	Laser	Bandpass filter	Brightness	Dyes with spectral overlap
A405	405	450/40	Dim	Other violet dyes (AmCyan, Qdot 605)
PerCP	488	670/30	Dim	PE, A594, PE-Cy5.5, APC, A700, A750
A488/ FITC	488	530/30	Bright	PerCP
PE-Cy7	561	780/60	Bright	PE, A594, PerCP, APC, PE-Cy5.5, A594, A700, A750
PE-Cy5.5	561	710/50	Bright	PE, A594, PerCP, PE-Cy7, APC, A700, A750
A594	561	610/20	Bright	PE, PerCP, PE-Cy5.5, PE-Cy7
PE	561	586/15	Very bright	PerCP, A594, PE-Cy7, PE-Cy5.5
A750	640	780/60	Dim	APC, A700, PerCP, PE-Cy7, PE-Cy5.5
A700	640	710/50	Moderate	APC, A750, PerCP, PE-Cy7, PE-Cy5.5
APC/ A647	640	670/14	Very bright	A700, A750, PerCP, PE-Cy7, PE-Cy5.5

4 Laser Strength on the Fortessa vs. the Calibur

The Calibur was one of the first widely utilized, commercially available flow cytometers, so there are limitations not only in the number of lasers and fluorochrome choices, but also with data processing. The Calibur processes data in analog mode, so the resolution is lower. In contrast, the Fortessa converts data digitally, so the resolution is much higher. The laser power is also quite different between the Calibur and Fortessa. For example, the blue laser on the Fortessa operates at 50 mW, and only at 15 mW on the Calibur. Another major difference is that biexponential scaling (see Chap. 3) isn't an option on the Caliber; fluorescence is run in log mode only. Given these differences, the data can look quite different when you compare the same set of stains on the Fortessa versus the Calibur (see Fig. 4.3). Both the CD4 and CD127 populations run ~1 log lower on the Calibur as compared to the Fortessa. As a result, the data appears more compressed (see far right dot plot). The Calibur range for fluorescence is 0–10⁴ whereas the Fortessa shows fluorescence below 0 and up to 10⁵; no negative decades are shown for the Calibur. As mentioned before, the laser strength is quite different, so it is not surprising that the MFIs are also much lower for CD4⁺CD127⁺ cells on the Calibur (51.7; 48%) versus the Fortessa (966; 54.7%).

5 High- vs. Low-Density Cell Markers and Fluorochrome Choice

We mentioned before that some fluorochromes are dim while others are bright. This is important to consider when choosing which one to pair with your antibody of interest. With a high-density marker such as CD4, there is quite a bit of flexibility.

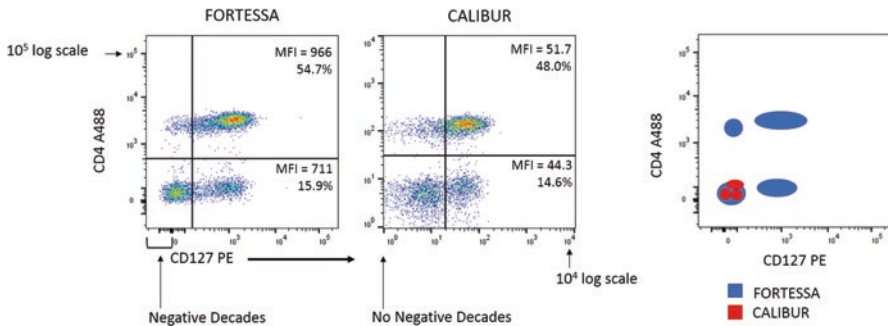


Fig. 4.3 CD4/CD127 expression on the BD LSRFortessa™ versus BD FACSCalibur™. Human whole blood was stained with hCD4 A488 and hCD127 PE, acquired on either the BD LSRFortessa™ or BD FACSCalibur™, and analyzed using FlowJo® software. The left and middle dot plots show CD4/CD127 expression on the Fortessa versus the Calibur, respectively. The far-right schematic shows how the data is more compressed on the Calibur

Refer to Fig. 4.4; the graphs show the level of CD4 expression on each fluorochrome associated with the four lasers. Across the board, the negative and positive populations are clearly expressed. As expected, the separation using CD4 A750 is not as desirable as compared to CD4 A700 or A647 as A750 is a weaker fluorochrome. The Median Fluorescence Intensity (MFI) values are also listed for CD4 on each of the fluorochromes. MFI values can be used to describe the expression levels of your protein of interest; however, this attribute of MFIs is situational and is largely restricted to protein expression on activated cells. For example, the MFI values for CD69, an activation marker on T cells, are high as these cells have been stimulated for 2 days with anti-hCD3/anti-hCD28 to induce T cell activation (refer to Fig. 3.2c). These high MFI values for CD69, along with the percentage, reveal high CD69 expression on activated T cells.

More routinely, MFI values reveal the signal intensity for a given fluorochrome. The MFI values in Fig. 4.4 reflect the signal intensity of CD4 expression (a T cell lineage marker) on naive T cells. Very bright dyes tend to have high MFI values (e.g., PE) whereas dim dyes have low MFI values (e.g., A405), reflecting the signal intensity of the fluorochrome. The true expression of CD4 is the same on all CD4⁺ T cells in Fig. 4.4; however, the intensity of the fluorochrome gives the impression of different levels of CD4 expression. Fluorochromes on the same PMT can also have different MFI values due to the nature of the dye. For example, APC and A647 cannot be used together as they have the same emission spectra and are detected using the same PMT. The MFI for A647 is 12,018 whereas the MFI of APC is around 4356. CD4 A647 is brighter, thus has a higher MFI value or signal intensity compared to CD4 APC.

Now we will discuss how a low-density marker such as CD127/IL-7R α is affected by fluorochrome choice. Refer to Fig. 4.5; the isotype/negative control is shaded in gray to give you an idea of the relative expression of CD127 on each fluorochrome across the four lasers. The MFI values are also listed for CD127 on each

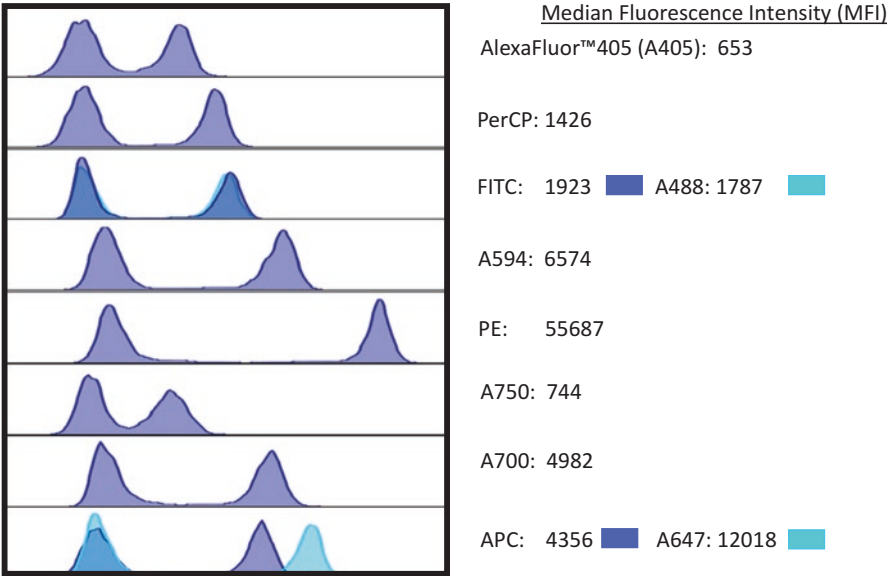


Fig. 4.4 Fluorochrome brightness with hCD4, a high-density marker. Human whole blood was stained with hCD4 A405, PerCP, FITC, A488, A594, PE, A750, A700, APC, or A647, acquired on the BD LSRFortessa™ and analyzed using FlowJo® software. A488 and A647 histograms are shown in light blue. MFI values for each fluorochrome are shown on the right

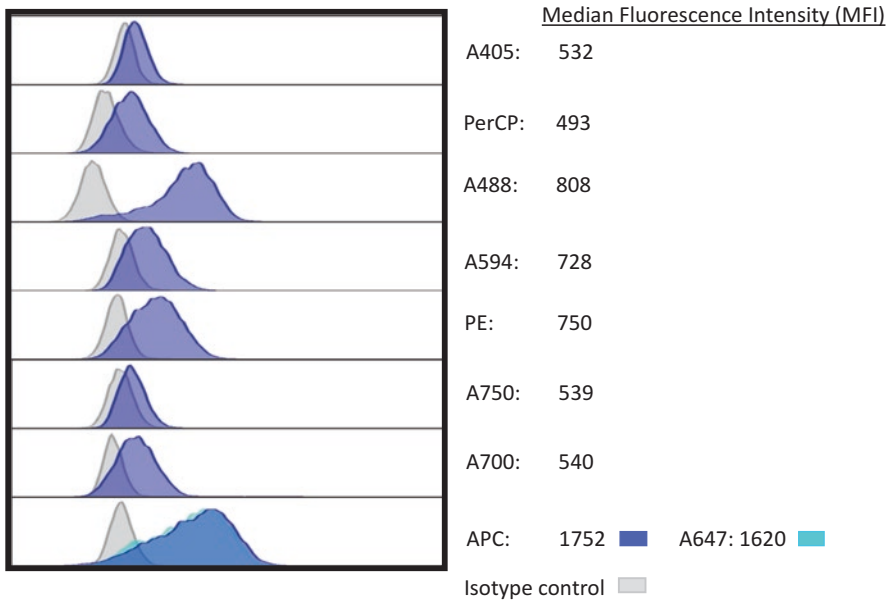


Fig. 4.5 Fluorochrome brightness with hCD127, a low-density marker. Human whole blood was stained with hCD127 A405, PerCP, A488, A594, PE, A750, A700, APC, or A647, acquired on the BD LSRFortessa™ and analyzed using FlowJo® software. The A647 histogram is shown in light blue. MFI values for each fluorochrome are shown on the right

Table 4.5 Summary of high- and low-density surface markers

Dye	High-density surface marker: CD4	Low-density surface marker: CD127	Brightness	Dyes with high spectral overlap
A405	Good	Poor	Dim	Other violet dyes
PerCP	Good	Poor	Dim	PE, APC/A647, A700, A750
A488/ FITC	Good	Good	Bright	PE, PerCP
A594	Good	Good	Bright	FITC, PE, PerCP
PE	Good	Good	Very bright	FITC, PerCP, A594
A750	Good	Poor	Dim	APC, A700, PerCP
A700	Good	Poor	Moderate	APC, A750, PerCP
APC/ A647	Good	Good	Very bright	A700, A750, PerCP

of the fluorochromes. The separation of CD127 relative to the isotype is poor on A405 and A750 as these are weak fluorochromes. In contrast, the staining intensity for CD127 is high on A488 along with PE and APC. This illustrates that it is important to use a *bright* fluorochrome on a *low*-density marker. Use of dim fluorochromes on low-density markers may result in poor detection or no detection at all if compensation values are high.

Here is a summary of what we just discussed (Table 4.5). CD4 is a safe bet on any of the fluorochromes since it is a high-density marker. In contrast, because it is a low-density marker, CD127 must be used in conjunction with a bright fluorochrome; A405, PerCP, A750, and A700 are not good options as they are dim fluorochromes. Again, when building your multicolor panel, you can consult both the Novus Biologicals Panel builder and Spectrum viewer online (novusbio.com).

In this chapter, we've discussed the various fluorochromes available on the market, and how to use online spectrum viewers to examine spectral overlap. We've compared fluorochrome choice on the Calibur versus the Fortessa, and how laser strength influences the appearance of your data. Finally, we've discussed the importance of pairing low-density markers with bright fluorochromes. Following these guidelines will aid in helping you design and run a successful flow cytometry experiment.

6 Materials

6.1 Flow Antibodies

- Human CD4 A488 (FAB3791G; R&D Systems)
- Human CD4 FITC (FAB 3791F; R&D Systems)
- Human CD4 PE (FAB3791P; R&D Systems)

- Human CD4 PerCP (FAB3791C; R&D Systems)
- Human CD4 A594 (FAB3791T; R&D Systems)
- Human CD4 APC (FAB3791A; R&D Systems)
- Human CD4 A647 (FAB3791R; R&D Systems)
- Human CD4 A700 (FAB3791N; R&D Systems)
- Human CD4 A750 (FAB3791S; R&D Systems)
- Human CD4 A405 (FAB3791V; R&D Systems)
- Human CD127 A488 (FAB360G; R&D Systems)
- Human CD127 FITC (FAB360F; R&D Systems)
- Human CD127 PE (FAB360P; R&D Systems)
- Human CD127 PerCP (FAB360C; R&D Systems)
- Human CD127 A594 (FAB360T; R&D Systems)
- Human CD127 APC (FAB360A; R&D Systems)
- Human CD127 A647 (FAB360R; R&D Systems)
- Human CD127 A700 (FAB360N; R&D Systems)
- Human CD127 A750 (FAB360S; R&D Systems)
- Human CD127 A405 (FAB360V; R&D Systems)

6.2 Staining Buffers/Fc Block Reagents

- Flow cytometry staining buffer (FC001; R&D Systems)
- Normal human IgG (1-001-A; R&D Systems)
- Mouse IgG
- Flow cytometry human lyse buffer (FC002; R&D Systems)

References

1. Bhattacharya B, Singha S, Basu S. Fluorescent nanosensors: rapid tool for detection of food contaminants. *Nanobiosensors*. 2017;841–74. <https://www.sciencedirect.com/book/9780128043011/nanobiosensors>
2. Grimm JB, et al. A general method to fine-tune fluorophores for live-cell and in vivo imaging. *Nat Methods*. 2017;14(10):987–94.
3. Pankchuk-Voloshina N, et al. Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright, photostable conjugates. *J Histochem Cytochem*. 1999;47(9):1179–88.
4. Maecker HT, Frey T, Nomura LE, Trotter J. Selecting fluorochrome conjugates for maximum sensitivity. *Cytometry A*. 2004;62A:169–73.

Chapter 5

Using the Process of Compensation to Prevent False Positive Data Caused by Fluorescence Spillover: A Practical Example



Christopher Hammerbeck, Christine Goetz, and Li Jen Peng

In nature, our eyes can distinguish between seven million different colors, many of which are unsurprisingly closely related shades. Our brain uses many spatial cues to separate objects of one shade from objects of different and/or related shades. A flow cytometer cannot separate colors with closely related spectrums on its own. If we do not instruct the cytometer as to how to identify and distinguish between colors with similar spectrums, flow cytometry plots become messy and uninterpretable. It is for this reason that, other than selecting the correct fluorochrome/antigen density combination for your antibody panel, correctly compensating for spectral overlap is one of the most important concepts to understand and perform correctly when establishing the parameters of a flow cytometry experiment. After all, where is the value in selecting a 10–12 color panel if many of the fluorochromes overlap and merge together and the cytometer is unable to distinguish one color from another?

1 Compensation: A Practical Example

Earlier we discussed the concept of spectral overlap or fluorescence spillover and why this happens in a more theoretical setting. Let's take a closer, more practical look at how this can affect flow cytometry results. Our first example clearly

Electronic Supplementary Material: The online version of this chapter (doi:[10.1007/978-3-319-98071-3_5](https://doi.org/10.1007/978-3-319-98071-3_5)) contains supplementary material, which is available to authorized users.

C. Hammerbeck · C. Goetz · L. J. Peng (✉)

Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA

e-mail: Chris.Hammerbeck@bio-technie.com; Christine.Goetz@bio-technie.com;

Li.Peng@bio-technie.com

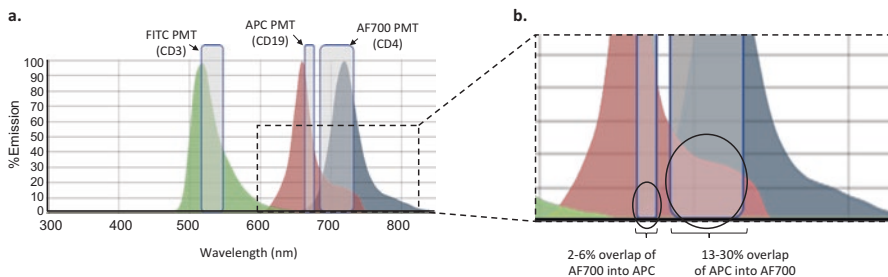


Fig. 5.1 Overlapping spectral emission profiles. **(a)** The spectral emission profiles for FITC (green), APC (red), and (A700) can be seen along with the areas of spectral overlap between APC and A700. The gray bars represent the detectors for FITC, APC, and A700 as indicated. **(b)** An enhanced view of the spectral overlap between APC and A700. Only 2–6% of the A700 emission spectrum overlaps into the APC detector while up to 30% of the APC emission spectrum overlaps into the A700 detector. Spectrum histograms were generated using the spectrum viewer available through Novus

demonstrates the need for compensating for fluorescence spillover by utilizes two types of human lymphocytes, T cells and B cells. By utilizing antibodies conjugated to fluorochromes that have high spectral overlap, we can make these cells appear to express surface markers that they do not actually express. In our example, we are staining for CD4⁺ T cells and CD19⁺ B cells. A quick review of published immunology literature or immunology textbooks will tell us that within the lymphocyte compartment, CD19 expression is restricted to B cells and CD4 expression is restricted to CD3⁺ T cells. B cells do not express CD4, nor do they express CD3 [1]. Thus, CD4 and CD19 expression are mutually exclusive on T and B cells, respectively. For the purpose of this example, we are going to stain human PBMCs with combinations of CD3 FITC, CD19 APC, and CD4 A700. In Fig. 5.1a we can see the predicted emission spectra for FITC (the green histogram), APC (the red histogram), and A700 (the blue histogram). The gray outlined boxes associated with each spectrum indicate the portion of each spectrum seen by the PMTs for each fluorochrome (FITC, APC, or A700). Notice how only a portion of each spectrum is detected by the cytometer. This is due to the presence of the bandpass filters associated with each PMT which limits the wavelength of light seen by each PMT. For example, FITC has an emission spectrum that spans roughly 480 nm to 640 nm. A common bandpass filter for the FITC PMT is the 530/30 filter. This filter allows light at 530 nm $\pm$ 15 nm on either side (for a total of 30 nm) to pass through to the FITC PMT. In other words, the FITC PMT will see light between 515 nm and 545 nm. By looking at Fig. 5.1, we can see that the FITC spectra is sufficiently distinct enough from APC and A700 that no spectral overlap occurs. Because no spectral overlap occurs between FITC and APC or FITC and A700, there should not be a need to compensate between FITC and APC or FITC and A700. In other words, fluorescence signals from CD3 FITC and CD19 APC are far enough apart on the emissions spectrum that all CD3⁺ T cells should be CD19 negative, and that all CD19⁺ B cells should be CD3 negative.

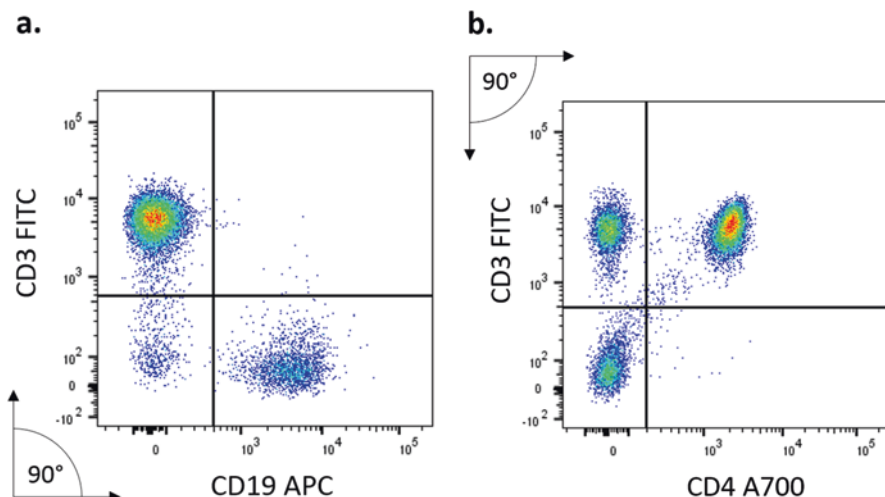


Fig. 5.2 APC and A700 do not present fluorescence spillover issues with FITC. Human PBMCs were stained with CD3 FITC, CD19 APC, and CD4 A700 antibodies. Plots show gated lymphocytes. The separation of the FITC emission spectrum with that of APC and A700 negates fluorescence spillover and compensation issues when FITC and APC (a) or FITC and A700 (b) are displayed on the same dot plot. Populations of cells within these plots form 90° angle and do not drift toward other quadrants

If the lymphocytes were to be stained with only CD3 FITC and CD19 APC antibodies (i.e., no CD4 A700 antibody has been added), as shown in the dot plot in Fig. 5.2a, three distinct populations of cells may be observed, forming a 90° angle. The CD19⁺CD3⁻ population in the lower right quadrant is B cells, the CD19⁻CD3⁺ population in the upper left quadrant is T cells, and the CD19⁻CD3⁻ population in the lower left quadrant is neither T nor B cells, but is in fact NK cells [1]. There are no double positive cells and the CD3 and CD19 staining is mutually exclusive. Similarly, if the lymphocytes were to be stained with only CD3 FITC and CD4 A700 antibodies (i.e., no CD19 APC antibody has been added), as shown in the dot plot in Fig. 5.2b, three distinct populations of cells are again observed, forming a 90° angle. The CD4⁺CD3⁺ population in the upper right quadrant is CD4⁺ T cells, the CD4⁺CD3⁻ population in the lower right quadrant is CD4⁺ T cells (likely CD8⁺ T cells), and the CD4⁻CD3⁻ population in the lower left quadrant is composed of both NK cells and B cells [1]. Appropriately, only one double positive cell population is observed: CD4⁺CD3⁺ T cells; neither B cells nor NK cells show positive staining for CD4 and CD3, and CD19 staining is mutually exclusive. A 90° angle is usually indicative of well-compensated samples. While this isn't always the case, it is a good initial criterion in which to visually inspect samples for appropriate compensation. Also keep in mind that when collecting the samples for the dot plots in Fig. 5.2, compensation had not yet been performed. All the fluorescence spillover values for the cytometer are displayed as zero. In this configuration, no spectral overlap will be corrected.

Now let's go back to the emission spectra histogram in Fig. 5.1a, focusing on the emission spectrums for APC (the red histogram) and A700 (the blue histogram). The peak emission wavelengths of these two spectrums are much closer (approximately 660 nm for APC and 720 nm for A700) and the right tail of the APC spectrum overlaps with the left tail of the A700 spectrum. This is where spectral overlap occurs. In Fig. 5.1b we zoom in on the APC and A700 spectrums. The 710/50 bandpass filter used to detect A700 allows light between 685 nm and 735 nm to pass through to the A700 PMT. The 670/14 bandpass filter used to detect APC allows light between 663 nm and 677 nm to pass through to the APC PMT. The 710/50 bandpass filter permits a fairly broad swath of light to reach the A700 PMT (between 685 nm and 735 nm). The emission wavelength for APC extends from roughly 600 nm to 750 nm, into the 685–735 nm range of detection utilized by the 710/50 bandpass filter to detect A700. The percent of emission for APC within the A700 PMT is between 13 and 30% which is substantial. This means that a significant portion of the APC spectrum will “bleed” into the A700 PMT and must be subtracted out of the A700 spectrum. In the above example, utilizing lymphocytes stained for CD4⁺CD3⁺ T cells and CD19⁺ B cells, we could predict that the fluorescence signal from the CD19 APC antibody will bleed into the CD4 AF700 PMT making the B cells appear to be significantly CD4 positive. By contrast, the 670/14 bandpass filter used to detect APC has a much narrower range of permitted light (between 663 nm and 677 nm only). The A700 spectrum extends from roughly 657 nm to 840 nm meaning that it too falls within the range of the 670/14 bandpass filter and will bleed into and be seen by the APC PMT. However, because the percent of A700 emission is so low between 663 nm and 677 nm (only 2–6%), a much smaller portion of the A700 spectrum must be subtracted out of the APC spectrum. Thus, we would anticipate that minor adjustments would have to be made in order to correct for the spillover of A700 into APC. Thus, we would predict that the CD4⁺ T cells would only appear to be slightly CD19 positive prior to compensation. It's important to remember that the percentages of spectral overlap reported by a spectrum viewer are estimated ranges of spillover for a given fluorochrome. The actual spillover percentage is only determined at the time compensation is performed during an experiment using the specific antibodies in your staining panel. The actual spillover values for every antibody are likely to be slightly different, even when conjugated to the same fluorochrome, but they should fall within the estimated ranges of spillover determined by the spectrum viewer.

As we will see shortly, the magnitude of the percent of emission of one fluorochrome seen by a second fluorochrome's detector is directly related to the size of the compensation values (i.e., the amount of one fluorochrome that needs to be subtracted from the second fluorochrome's detectable spectrum) for those two fluorochromes. While this may seem complicated, it should make sense by the time you've completed this chapter.

We've just finished explaining why spectral overlap occurs in respect to fluorochrome emission spectrums and bandpass filters, but what does that look like on a flow plot? As mentioned in Fig. 5.2, the FITC and APC, and FITC and A700 spectrums don't overlap; however, the spectrums for APC and A700 do overlap. In

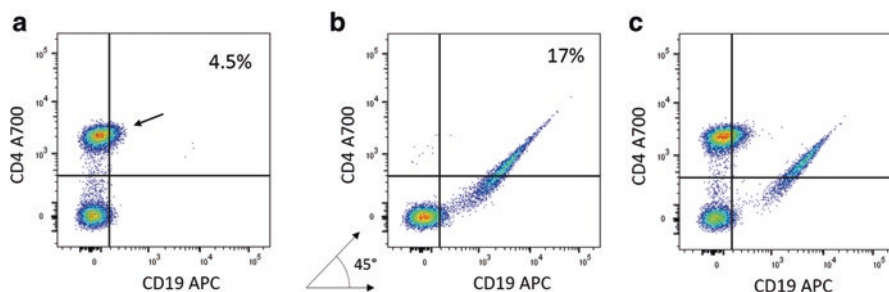


Fig. 5.3 Fluorescence spillover between APC and A700 causes major and minor compensation issues. Human PBMCs were stained with combinations CD3 FITC, CD19 APC, and CD4 A700 antibodies. Plots show gated lymphocytes. **(a)** Cells were stained with CD3 FITC and CD4 A700 antibodies. No CD19 APC antibody was included. The small percentage of spectral overlap of A700 into APC causes the CD4 A700 population to slightly drift toward the right (increased APC positive fluorescence). **(b)** Cells were stained with CD3 FITC and CD19 APC antibodies. No CD4 A700 antibody was included. The large percentage of spectral overlap of APC into A700 causes the CD19 APC population to significantly drift up (increased A700 positive fluorescence). **(c)** Cells were stained with all three antibodies and the effects of spillover of APC into A700 and A700 into APC can be seen on the same plot

Fig. 5.3a we have cells stained with CD3 FITC and CD4 A700 and are showing A700 fluorescence (Y-axis) versus APC fluorescence (X-axis). No CD19 APC antibody has been added to these samples. However, notice that the CD4⁺ T cell population in the upper left quadrant is drifting slightly to the right. If we set the CD19 negative gate on the CD4⁻ population in the bottom left quadrant such that 4–5% of those cells are CD19 positive, 17% of the CD4⁺ population appears to express CD19 (black arrow), albeit at low levels. We know from published literature that CD4⁺ T cells do not express CD19. Rather, what we are seeing is the slight bleed over of A700 into the APC PMT. On the other hand, when we look at the spillover of APC into the A700 PMT shown in Fig. 5.3b, we can see that now we have a major problem. Here we have cells stained with CD3 FITC and CD19 APC and are showing A700 fluorescence (Y-axis) versus APC fluorescence (X-axis). No CD4 A700 antibody has been added but because a significant portion of the APC spectrum overlaps with the A700 spectra, and the percentage of emission of APC is fairly substantial between 685 nm and 735 nm (the wavelengths seen by the A700 PMT), the CD19 B cells appear to also be CD4 positive. Moreover, instead of the two populations forming a straight horizontal or vertical line, we have a line that is closer to a 45° angle. Finally, in Fig. 5.3c, we have cells stained with all three antibodies, CD3 FITC, CD4 A700, and CD19 APC. The CD19 APC signal is strong enough that it bleeds over into the A700 PMT so that most of the CD19⁺ B cells appear to express an intermediate amount of CD4 and, even though the spillover of A700 into the APC PMT is so minimal, the CD4⁺ T cells still appear to express a low amount of CD19. These results meet what we predicted after comparing the spectral emission plots earlier in Fig. 5.1.

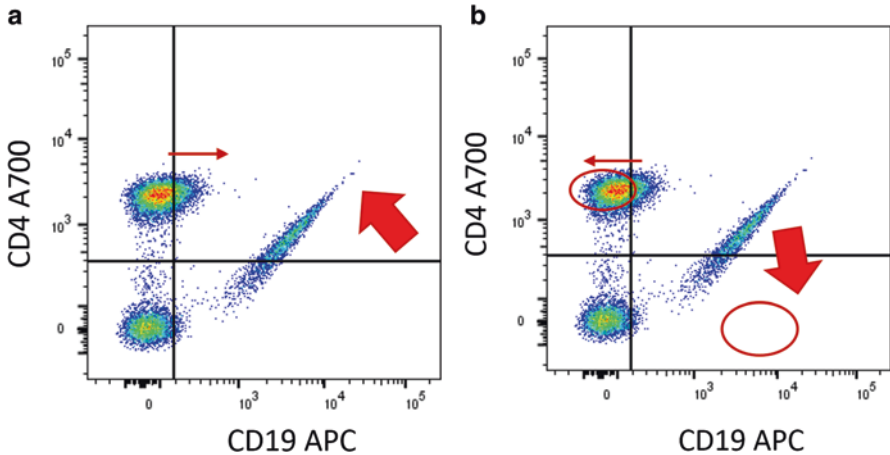


Fig. 5.4 How will compensation affect the position of cell populations? (a) Spectral overlap between A700 and APC results in a slight drift of the CD4 A700 population toward the right (increased APC positive fluorescence; represented by the thin red arrow) and a significant upward drift of the CD19 APC population (increased A700 positive fluorescence; represented by the thick red arrow). (b) After applying compensation, the correct placement of the CD4A700 and CD19APC populations is shown by the red ovals and the degree and direction that the populations should shift is represented by the thin (slight) and thick (significant) red arrows

We still have three distinct populations of cells, but the populations aren't exactly where they should be. When looking closely at the dot plot in Fig. 5.4a, it is apparent that the CD4⁺ T cell positive population in the top left quadrant drift slightly toward the right. This is due to the slight amount of spillover from A700 into the APC PMT and is represented by the thin red line. By comparison, the amount of spillover of APC into the A700 PMT is substantial causing the CD19⁺ B cell positive population in the lower right quadrant to drift up and left into the upper right quadrant which is represented by the thick red arrow. On the dot plot in Fig. 5.4b the red circles indicate where the populations should appear after properly compensating for spillover between APC and A700. The CD4⁺ T cell positive population in the top left quadrant should shift slightly to the left and the CD19⁺ B cell positive population should shift down and to the right, into the lower right quadrant.

To adjust the position of these populations into the correct quadrants of the plot, it is critical to first define each fluorochrome as a solitary signal. Once that is accomplished and a mathematical matrix has been established by the cytometer, it will be able to compensate for the presence of one fluorochrome's emission spectrum spilling into another fluorochrome's emission spectrum. Two things are required to perform compensation: (1) a sample of cells stained with fluorochrome-conjugated antibodies and (2) compensation beads or cells (described below in Sect. 3.6 of this chapter) individually stained with the same fluorochrome-conjugated antibodies. As mentioned in Chap. 3, Sect. 3.1.2, a small number of cells are collected first and are used to establish the voltage setting for each PMT. Compensation beads are then

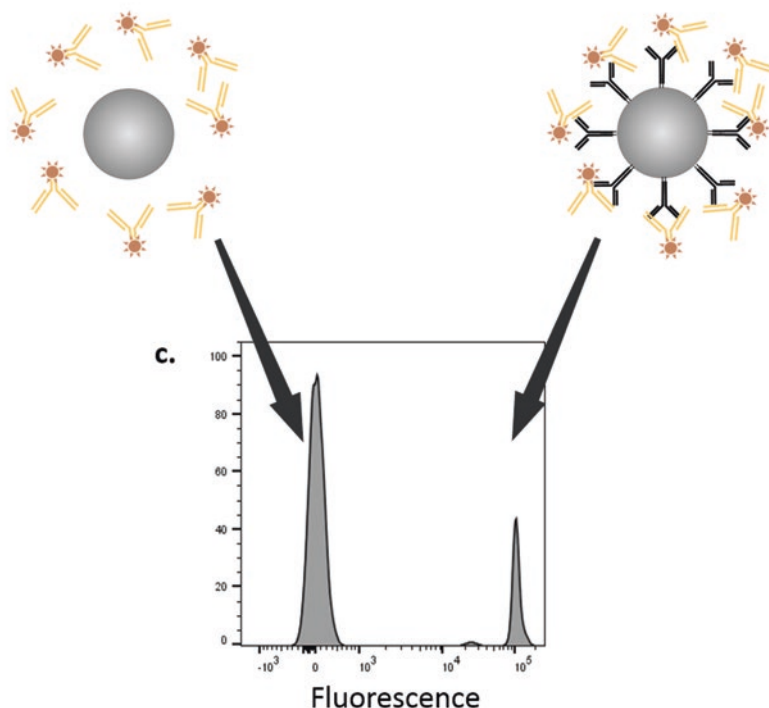
a. Negative Control Beads**b. Positive Control Beads**

Fig. 5.5 Compensation beads. Compensation beads consist of two bead populations: a negative control bead (**a**) that will not bind flow cytometry antibodies and a positive control bead (**b**) that will bind flow cytometry antibodies. When used together and the compensation bead fluorescence is plotted as a histogram, the negative control bead shows up as the negative fluorescence peak and the positive control bead shows up as the positive fluorescence peak

collected, using the predetermined voltages, to establish each fluorochrome as a solitary signal.

In order to accomplish this, compensation beads must be single stained (Fig. 5.5) using the antibodies selected for the panel in the experiment. Each tube of compensation beads that are prepared must include beads that have been coated with antibody specific to the cell used in the experiment, as well as negative uncoated beads (Fig. 5.5a). The coated beads are positive controls and are specific for antibodies made in a given species such as mouse, rat, or hamster. It is important to select the bead that is specific for the species of cells being used in the experiments in order for the flow cytometry antibody to bind. For example, if using an antibody made in mice, select compensation beads that are specific for mouse antibodies. The second type of beads in the compensation bead mix is a negative control bead (Fig. 5.5b). These are beads that are not coated with any antibody and will not bind to flow cytometry antibodies. When mixed together, these beads will define a negative

Table 5.1 Compensation bead setup

Tube	Fluorochrome	Antibody
1	None	Unstained beads
2	FITC	CD3
3	APC	CD19
4	A700	CD4

unstained population and a positive antibody-labeled population (Fig. 5.5c). In the example of FITC, APC, and A700 compensation, there will be one tube per fluorochrome and one tube of unstained beads for a total of four tubes. Tube 1 will contain the unstained beads, Tube 2 will have beads stained with CD3 FITC, Tube 3 will have beads stained with CD19 APC, and Tube 4 will have beads stained with CD4 A700 (Table 5.1).

Prior to collecting the compensation beads, a small number of stained cells are acquired to establish the voltage settings for each PMT, or each fluorescence channel, being used. The voltages are adjusted for each fluorochrome (in this case FITC, APC, and A700) such that the negatively stained population for each color falls, ideally, between 10^0 and 10^2 on the log scale. Keep in mind that the process of compensation can result in a slight dimming of each fluorochrome so positioning the negative population closer to 10^2 (i.e., setting the voltage a little higher) can help prevent the loss of too much fluorescence signal. This has already been done for the dot plots shown in Figs. 5.2 and 5.3. To be clear, voltage settings for each fluorochrome are set using cells, not the single-stain compensation beads. Another start-to-finish example of this process is demonstrated in the Supplemental Online Video.

Figure 5.6 shows the fluorescence staining patterns of the compensation beads prior to performing compensation. Using the FITC single-stain compensation bead, a positive FITC signal can be detected in the FITC channel (Fig. 5.6a) but not in the A700 (Fig. 5.6b) or APC (Fig. 5.6c) channels. Using the APC single-stain compensation beads, there is no positive signal in the FITC channel (Fig. 5.6d) but we can see a positive signal in both the APC channel (Fig. 5.6f) and the A700 channel (black arrow) (Fig. 5.6e). Lastly, using the A700 single-stain compensation bead, there is no positive signal in the FITC channel (Fig. 5.6g) but now a positive signal can be seen in the A700 channel (Fig. 5.6h). In the APC (Fig. 5.6i) channel, while a distinct positive peak does not exist, a slight shoulder can be seen on the right side of the APC peak (black arrow). This nicely illustrates the fluorescence spillover of APC into the A700 PMT that was seen using PBMCs.

When performing compensation, run each single-stain compensation bead sample separately. If using a cytometer that is run by FACSDIVA™ software, such as the BD FACS Aria™, BD LSRFortessa™, BD FACSCanto II™, or BD LSR II™, running compensation beads separately allows the AutoCompensation software to identify a true negative and true positive signal for all fluorochromes being used. The software will then mathematically determine what percentage of the spectral emission of one fluorochrome needs to be subtracted from the spectral emission from another fluorochrome. The process will be completed for every combination

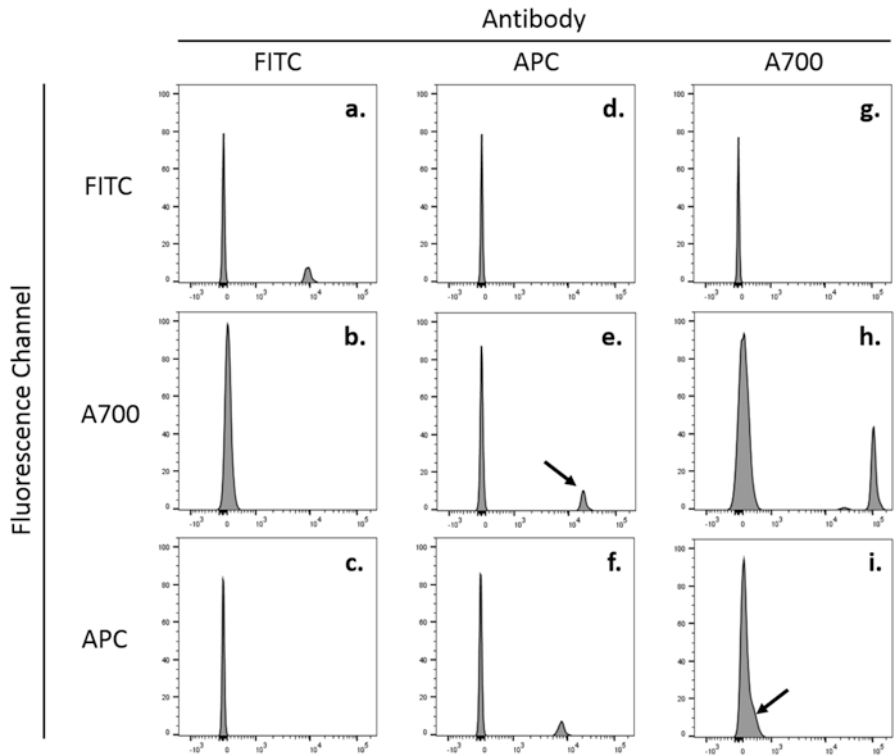


Fig. 5.6 Fluorescence profiles of CD3 FITC, CD19 APC, and CD4 A700 compensation beads BEFORE compensation has been applied. Positive control and negative control compensation beads were incubated with a CD3 FITC antibody (a, b, c), a CD19 APC antibody (d, e, f), or a CD4 A700 antibody (g, h, i). No compensation was performed, and the fluorescence profiles of beads stained with these antibodies were plotted against each channel used for the experiment: FITC (a, d, g), A700 (b, e, h), and APC (c, f, i). Beads stained with the CD3 FITC antibody are only detected in the FITC channel (a). Beads stained with the CD19 APC antibody were detected in the APC channel (f) and in the A700 channel (e). Beads stained with the CD4 A700 antibody were detected in the A700 channel (h) and a small shoulder (black arrow) was seen in the APC channel (i)

of fluorochromes being used. Performing an 8-color flow experiment would result in 64 potential fluorochrome combinations making the AutoCompensation feature both useful and necessary. In this example, we are only using three fluorochromes so there are six possible combinations for which spectral overlap needs to be evaluated and corrected: APC - %FITC (read APC minus percent FITC), A700-%FITC, FITC-%APC, A700-%APC, FITC-%A700, APC-%A700.

Figure 5.7a shows the spillover values for the cytometer prior to performing compensation. All the values are set to zero. With this configuration, no spectral overlap is being identified and no spectral overlap will be corrected. When put into a table form, the fluorescence spillover that occurs across the various combinations of fluorochromes is referred to as a spillover matrix that can be seen in Fig. 5.7b.

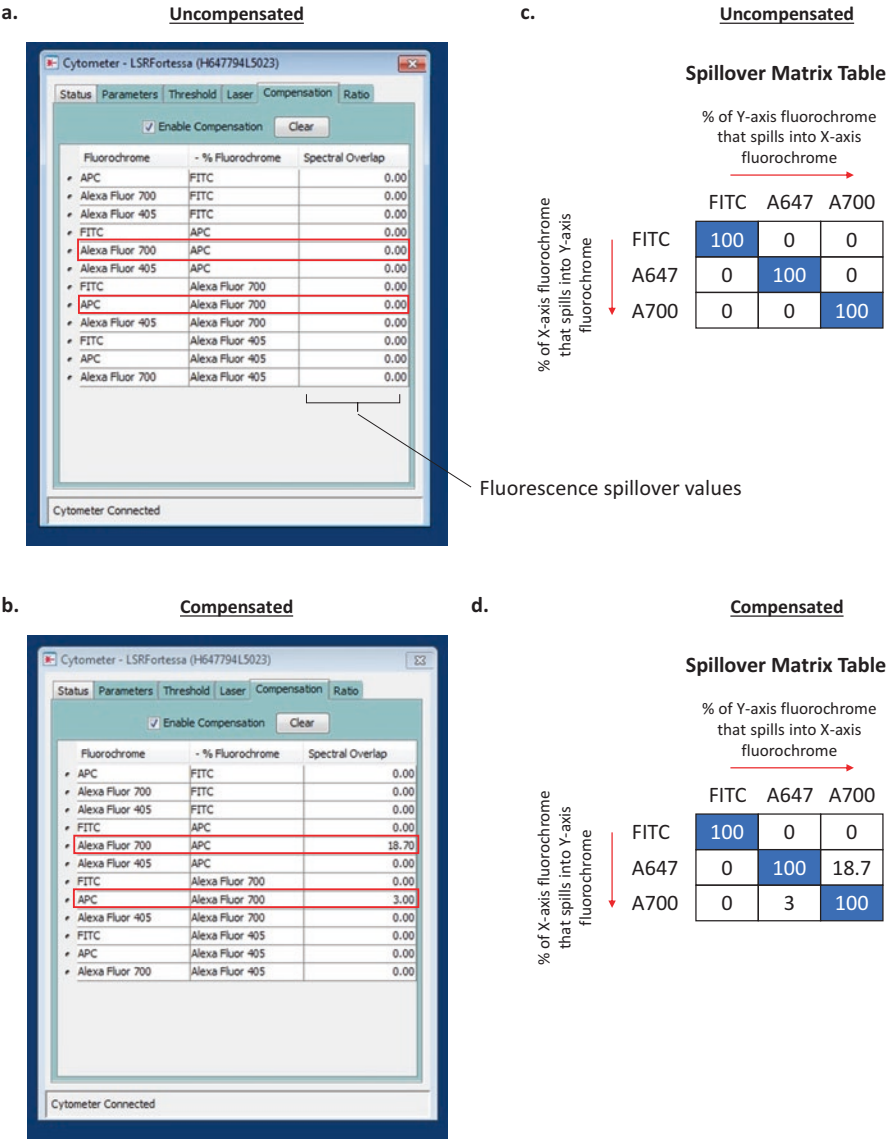


Figure 5.7c shows the spillover values for the cytometer after performing AutoCompensation and again, when put into a spillover matrix table in Fig. 5.7d. The FACSDIVA™ software mathematically converts the spillover matrix values into another set of numbers, an actual compensation matrix, that tell the cytometer how much of each fluorochromes emission spectrum to subtract from all fluorochromes within the antibody panel. You will never see the compensation matrix values, even if you perform compensation manually; only the spillover values. Earlier it was stated that the magnitude of the percent of emission of one fluorochrome seen by the PMT for a second fluorochrome is directly related to the size of the spillover values for those two fluorochromes (i.e., the amount of one fluorochrome that needs to be subtracted from the second fluorochrome). The change in spillover values seen between Fig. 5.7b, d illustrated this concept. Because the spillover of APC into the A700 PMT is significant (the percent emission of APC in the wavelengths seen by the A700 PMT is predicted to be between 13 and 30%, Fig. 5.1), the size of the actual spillover value obtained after compensation should fall within this range. At 18.70, it does. Consequently, the amount of APC that will need to be subtracted from A700 is also fairly large. In comparison, the spillover of A700 into the APC PMT is slight (the percent emission of A700 in the wavelengths seen by the APC PMT is predicted to be between 2 and 6%), the size of the actual spillover value obtained after compensation should also be small. At 3, it is small and consequently means that the amount of A700 that will need to be subtracted from APC is fairly small. Notice that because different excitation lasers are used to excite FITC (488 nm blue laser) and APC and A700 (640 nm red laser), the FITC spectrum is distinct from both APC and A700, the spillover values obtained for those combinations remained zero before and after compensation.

A few important details need to be addressed here including: (1) how the emission percentage of a fluorochrome affects the degree of fluorescence spillover, (2) how that's different than the spectrum of one fluorochrome simply overlapping with the spectrum of another fluorochrome at a wavelength seen by the PMTs for those fluorochromes, and (3) how fluorochrome brightness all affect spillover values.

In this scenario, the spectrums for both APC and A700 overlap with each other (Fig. 5.1). From Chap. 4 we also know that APC is inherently brighter than A700 the same way a 100-watt lightbulb is brighter than a 60-watt lightbulb; both are able to illuminate a space but one is much brighter. The PMT for APC can see A700 and the PMT for A700 can see APC, yet the spillover of APC into A700 is more problematic and results in a larger spillover value than the spillover of A700 into APC. Why? It has to do with the intensity of the APC signal seen by the A700 PMT compared to the intensity of the A700 signal seen by the APC PMT. The wavelength of the APC emission spectrum at which the APC signal is at its peak intensity, or 100% emission, is around 650 nm. At no other wavelength will APC fluoresce as brightly as it does at 650 nm. The APC PMT sees APC at between 663 nm and 677 nm only where it's at 40–75% of its peak intensity. By comparison, within that narrow wavelength, A700 is only at 2–6% of its peak intensity. In other words, A700 is very dim between 663 nm and 677 nm.

Imagine a child has two lights, one with a 20-watt red lightbulb and one with a 100-watt blue lightbulb. Both lights have a dimmer switch and the child is shining the lights on a wall trying to make the wall turn purple. The child dims the red lightbulb until it is barely visible (this represents A700) but leaves the blue lightbulb at its full intensity (this represents APC). The red lightbulb lacks sufficient intensity to compete with the blue lightbulb so the wall looks blue. In a similar way, A700 affects the APC signal very little and has a very low compensation value. By comparison, at the wavelengths seen by the A700 PMT (685 nm to 735 nm), APC is at approximately 30% of its peak intensity. Even though A700 is at its peak intensity within this range, A700 is sufficiently dim that an APC signal at 30% emission intensity affects the A700 signal significantly and results in a higher compensation value. In this scenario, imagine that the child is now shining the 20-watt red lightbulb at full intensity but dims the 100-watt blue lightbulb by almost half. Now the intensity of the red and blue lights is more comparable. You'd still be able to identify red and blue lights on the wall but it would be more difficult to determine where the red light began and the blue light ended.

We can now see how compensation affects the fluorescence spillover between APC and A700 by looking at the compensation beads in Fig. 5.8. Compare these results with the results shown in Fig. 5.6. Nothing has changed with the detection of the FITC single-stain compensation beads. A positive signal can be seen in the FITC channel (Fig. 5.8a) but not in the A700 (Fig. 5.8b) or APC (Fig. 5.8c) channels. Using the APC single-stain compensation beads, a positive signal can be seen in the APC channel (Fig. 5.8f) but not in the FITC channel (Fig. 5.8d). Furthermore, the positive signal that was once present in the A700 (Fig. 5.8e) channel has disappeared (black arrow). Lastly, using the A700 single-stain compensation bead, there is no longer a positive signal in the FITC (Fig. 5.8g) channel, the small shoulder on the side of the APC peak has disappeared (black arrow) (Fig. 5.8i) and the only positive signal can be seen in the A700 channel (Fig. 5.8h).

Once compensation has been performed, it is easy to see how removing fluorescence spillover affects the expression of CD4 on B cells and CD19 on T cells. We know from Fig. 5.1 that the FITC spectrum is sufficiently distinct enough from APC and A700 that no spectral overlap occurs. Because no spectral overlap occurs between FITC and APC and FITC and A700, the spillover values remain zero for those fluorochrome combinations. Therefore, the dot plots showing CD3 FITC versus CD19 APC (Fig. 5.2a) or CD3 FITC versus CD4 A700 (Fig. 5.2b) will not change after performing compensation.

As shown in Fig. 5.9a, cells were stained with CD3 FITC and CD4 A700 and are showing A700 fluorescence (Y-axis) versus APC fluorescence (X-axis). No CD19 APC antibody has been added to these samples. Prior to compensation, the CD4⁺ T cell population in the upper left quadrant drifted slightly to the right with 17% of the CD4⁺ T cells appearing to express CD19 (Fig. 5.3a). After compensation, the CD4 A700 population longer drifts slightly toward the upper right quadrant and no longer appears to express low levels of CD19. Again, the minor shift in the population reflects the small spillover value applied due to the relatively minor amount of A700 that needed to be subtracted out of the APC PMT. More impressively, with the compensation matrix applied, the B cell population is now where it belongs, down in the

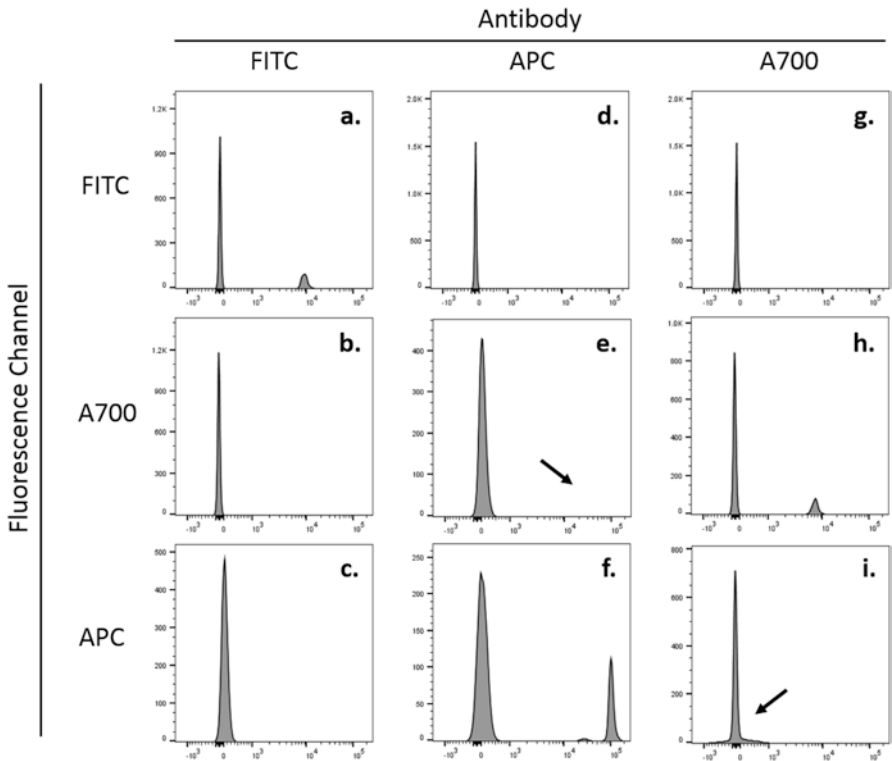


Fig. 5.8 Fluorescence profiles of CD3 FITC, CD19 APC, and CD4 A700 compensation beads AFTER compensation have been applied. Positive control and negative control compensation beads were incubated with a CD3 FITC antibody (a, b, c), a CD19 APC antibody (d, e, f), or a CD4 A700 antibody (g, h, i). Compensation was performed and then the fluorescence profiles of beads stained with these antibodies were plotted against each channel used for the experiment: FITC (a, d, g), A700 (b, e, h), and APC (c, f, i). Beads stained with the CD3 FITC antibody are still only detected in the FITC channel (a). Beads stained with the CD19 APC antibody are now only detected in the APC channel (f). Beads stained with the CD4 A700 antibody are now only detected in the A700 channel (h). Notice the absence of the A700 positive shoulder (black arrow) in the APC channel (i) and the absence of the APC positive peak (black arrow) in the A700 channel (e)

lower right quadrant (Fig. 5.9b). Prior to compensation (Fig. 5.3b), the CD19⁺ B cell population and the CD4⁺CD19⁻ population formed a line that was close to 45 degrees, making it appear that the CD19⁺ B cells were expressing intermediate to high levels of CD4. After the APC spectral overlap was subtracted out of the A700 PMT during compensation, the B cells no longer appear to express CD4 and the CD4⁺CD19⁻ population and CD19⁺ B cell population form a straight horizontal line. Finally, the problem has been solved. In Fig. 5.9c, cells were stained with all three antibodies, CD3 FITC, CD4 A700, and CD19 APC. The three distinct populations of cells form a 90° angle compared to what was shown in Fig. 5.3c. The B cells no longer appear to express CD4 but express only CD19 and the T cells express only CD4 and not CD19, consistent with published literature.

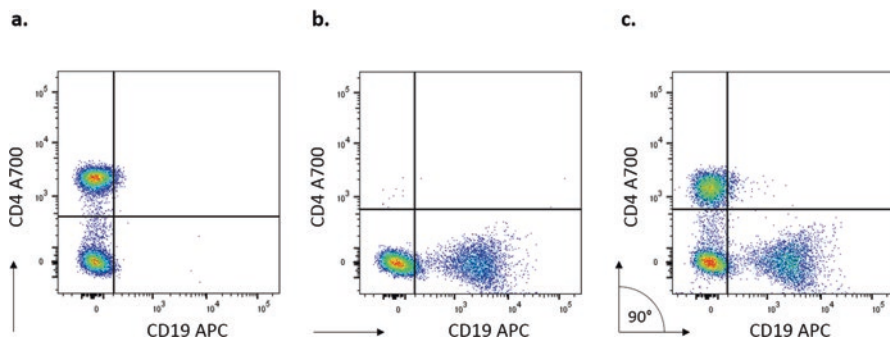


Fig. 5.9 Compensation for spectral overlap between APC and A700 resolves major and minor fluorescence spillover issues. Human PBMCs were stained with combinations CD3 FITC, CD19 APC, and CD4 A700 antibodies and compensation was performed. Plots show gated lymphocytes. (a) Cells were stained with CD3 FITC and CD4 A700 antibodies. No CD19 APC antibody was included. The CD4 A700 population no longer slightly drifts toward the right (increased APC positive fluorescence). (b) Cells were stained with CD3 FITC and CD19 APC antibodies. No CD4 A700 antibody was included. The CD19 APC population no longer significantly drifts up (increased A700 positive fluorescence). (c) Cells were stained with all three antibodies. All three populations are now properly aligned

2 Manual Compensation by Comparing the Median Fluorescence Intensity of Cell Populations

While more modern cytometers running FACSDIVA™ offer an AutoCompensation feature, other instruments such as the BD FACSCalibur™ do not offer this feature because they are run off different software, such as BD CellQuest™, which does not offer this function. The compensation process in the BD CellQuest™ program must be performed manually either by typing spillover values into boxes pertaining to various fluorochrome combinations or by gradually increasing or decreasing spillover values using up/down arrows or by manipulating a sliding scale in the Compensation window. This instrument only supports 4-colors (named FL1, FL2, FL3, FL4) which are excited by only two lasers making the number of potential fluorochrome combinations more manageable. However, one potential pitfall in performing manual compensation is that populations visually appearing to be correctly positioned (i.e., 90° angles versus 45° angles between populations) may not be mathematically compensated. A better way to insure proper manual compensation is by comparing the median fluorescence intensity (MFI) of negatively stained and positively stained populations using single-stain compensation beads.

This concept is illustrated in Fig. 5.10 using the example of CD4 A700 versus CD19 APC. In Fig. 5.10a, b, compensation is used to correct for the spillover of A700 into APC. Figure 5.7 indicates that this value should be fairly small, near 3. We begin by collecting the A700 single-stain control beads and find the two cell populations along the Y-axis, a A700-negative population in the lower left quadrant

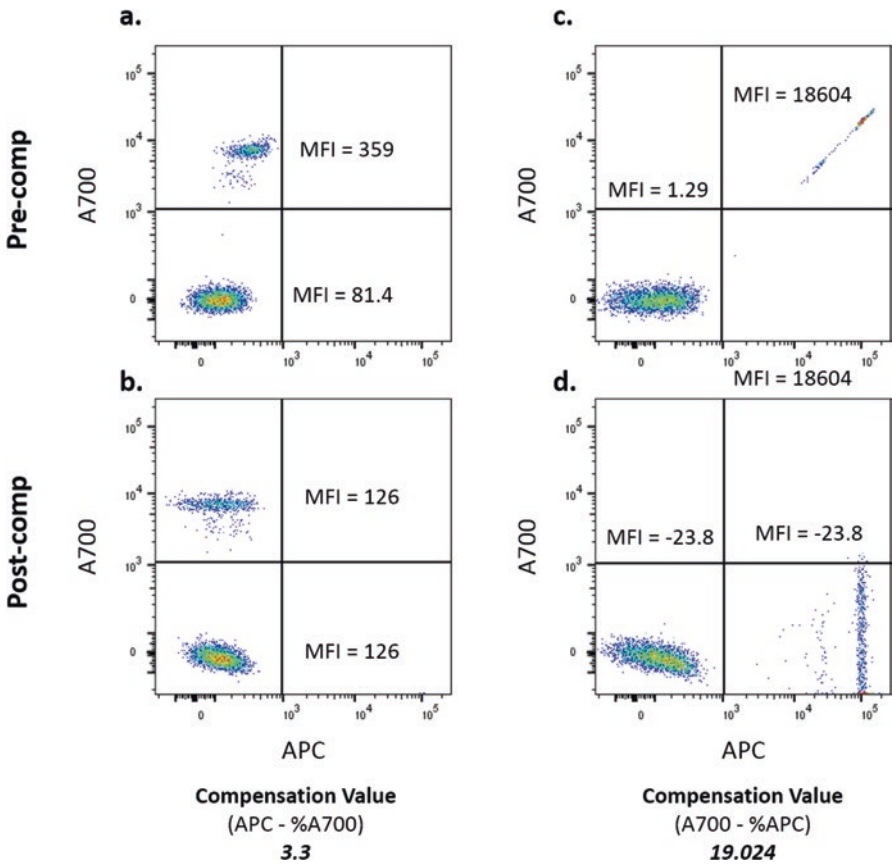


Fig. 5.10 Manual compensation by comparing MFI. Positive control and negative control compensation beads were incubated with a CD4 A700 antibody (**a** and **b**) or a CD19 APC antibody (**c** and **d**) and A700 versus APC fluorescence was compared. The MFI for both fluorescence negative and fluorescence positive events was determined for each fluorochrome. To compare spillover of A700 into APC (**a**), the MFI for APC fluorescence was determined. The compensation value for APC-%A700 was adjusted until the MFI for APC fluorescence for the fluorescence negative and fluorescence positive events was equal (**b**). To compare spillover of APC into A700 (**c**), the MFI for A700 fluorescence was determined. The spillover value for A700-%APC was adjusted until the MFI for A700 fluorescence for the fluorescence negative and fluorescence positive events was equal (**d**)

and an A700 positive population in the upper left quadrant (Fig. 5.10a). It's clear that the A700 positive population drifts toward the right, appearing slightly APC positive. If we compare the median fluorescence intensity (MFI) of APC fluorescence (X-axis) for each population, we find that the MFI for the A700 negative population is 81.4 and the MFI for the A700 positive population is 359. When properly compensated, these MFI values should be approximately equal. Adjusting the spillover value for APC minus A700 to 3.3 results in an MFI of 126 for APC fluorescence for both the A700-negative and A700 positive populations. Next, the

spillover values of APC into A700 will be manually adjusted (Fig. 5.10c, d). Figure 5.7 indicates that this value should be fairly large, near 18 or 19. After APC single-stain control beads have been collected, two cell populations can be found along the X-axis: (1) an APC-negative population in the lower left quadrant and, because of the high spillover of APC into the A700 PMT, (2) an APC positive population that has drifted into the upper right quadrant (Fig. 5.10c). If we compare the MFI of A700 fluorescence (Y-axis) for each population we find that the MFI for the A700-negative population is 1.29 and the MFI for the A700 positive population is 18,604, which is drastically different.

When properly compensated, these MFI values should be approximately equal. Adjusting the compensation value for A700 minus APC to a spillover value of 19.024 results in an MFI of -23.8 for the A700 fluorescence for both the APC-negative and APC positive populations. Even though the MFI obtained for A700 fluorescence is in the negative decades, this is ok, albeit not optimal. This can happen with dimmer fluorochromes with longer wavelengths. The widening of the A700 positive population is also normal and is the result of small mathematical errors in measuring fluorescence. These errors become accentuated when making large spillover adjustments (APC spilling over into A700) between a bright (APC) and dim (A700 color). The specific details are beyond the scope of this book, but for details on this and everything else related to flow cytometry compensation, Mario Roederer's website is a fantastic resource [2]. Remember, the PMT voltages were set using the actual cells that will be collected meaning that the populations will look correct when they are collected. Simply turning up the voltage for the A700 PMT to bring the MFI into the positive decades can actually make compensation worse. This will be discussed in the “*Considerations*” section at the end of this chapter.

It should be noted that the AutoCompensation process is not always perfect and samples can end up being overcompensated as well as undercompensated. Along with visually evaluating the position of the cell populations after compensation has been performed, comparing MFIs as described above can be a good way to assess the accuracy of the AutoCompensation process. How to resolve over- and under-compensation issues is another area of debate among flow cytometrists. Some are adamant that once compensation has been performed and spillover/compensation matrices have been established, those matrices should not be further manipulated just because a cell population looks wrong. They would argue that the steps leading up to compensation (panel design, antibody titration, bead, and/or cell staining) need to be evaluated to correct for any visual abnormalities resulting from the compensation process. Clearly, if over- and under-compensation becomes a repeated issue, these upstream processes should be evaluated first. Other cytometrists argue that if a population is clearly over- or under-compensated, spillover/compensation matrices can be “reasonably” tweaked to correct for these issues. In this case, “reasonable” is the key word. Once compensation has been performed, the position of cell populations should not be adjusted by eyeball simply because they look wrong. We mentioned that there are likely fluorescence spillover issues when cells appear as diagonal lines on two-color flow plots. However, some populations will appear

more diagonal than others, even when properly compensated. Tweaking the position of these populations simply because they look wrong to the eye would just be incorrect. It is necessary to understand the expression pattern of a protein of interest on a given cell population before attempting to modify compensation values. The best way to do this is by comparing the staining pattern of the protein using multiple antibody panels. Whenever possible, perform small, preliminary experiments using fluorochromes that do not require compensation to establish the expression pattern of a protein of interest. In this way you can be confident that fluorescence spillover is not affecting the detection of the protein of interest. The staining pattern should be similar using larger panels that require compensation although the staining intensity may be slightly reduced by the compensation process. Only after establishing these baselines could a “reasonable” attempt be made at tweaking spillover/compensation matrices after compensation has been performed.

3 Considerations When Performing Compensation

3.1 *Using the Same Compensation Settings for Multiple Experiments*

Spillover values between experiments can vary so be careful to not blindly rely on the compensation values from one experiment being able to correctly compensate samples in another experiment. Each antibody is unique, each antibody conjugation is unique, and each antibody combination is unique. Similarly, the voltages used for a given fluorochrome can change from one experiment to the next and this can have a profound impact on compensation values. For example, in the AutoCompensation example in Fig. 5.7, the spillover values for A700-%APC and APC-%A700 were 18.7 and 3, respectively. In the Manual Compensation example in Fig. 5.10, using the same CD4 A700 and CD19 APC antibodies and the same voltage settings, the spillover values for A700-%APC and APC-%A700 were comparable at 19.024 and 3.3, respectively. Similarly, if we keep the A700 and APC voltage settings the same but use a different combination of antibodies, CD163 A700 and HLA-DR APC, we find that the spillover values for A700-%APC and APC-%A700 are again comparable at 18.04 and 3.34, respectively. As you can see, when used at the same voltage, the spillover values between the same combinations of fluorochromes are fairly concordant. All three sets of numbers are very close and using these sets of values interchangeably would result in dot plots that look similar. However, beware that small changes in spillover values can result in incorrect compensation and can lead to potentially false positive or false negative data. Now consider what happens if we use those same CD4 A700 and CD19 APC antibodies but change the APC and A700 voltages. In Fig. 5.10, the voltage for APC was set to 580 and the voltage for A700 was set to 620 and the spillover values for A700-%APC and APC-%A700 were 19.024 and 3.3, respectively. If the voltage setting for APC was changed to 604 and

the voltage for A700 was changed to 720 now the compensation values for A700–%APC and APC–%A700 become 129.17 and 0.49, respectively. In other words, spillover values from one experiment are no longer compatible if voltages are changed between experiments.

From a practical standpoint, many investigators will reuse compensation settings from one experiment to the next. To be clear, for this to work, the same antibody panel must be used from one experiment to the next and all PMT voltage settings must remain unchanged. Many times, this is done when using inbred mouse models as they are less subject to experiment-to-experiment variability than outbred animal models or human cells. Further, this is more likely to be successful when studying discrete cell populations that are identified by congenic or lineage markers than examining the expression of variably, and sometimes weakly, expressed proteins. This is because slight differences in spillover values when compensating cell populations defined by lineage markers won't likely affect the ability to distinguish those populations. Consider the T cell / B cell example shown earlier. Even when the samples were uncompensated, it was still possible to separate B cells and T cells. When proteins with variable expression are involved, small changes in spillover values could be the difference between detecting and not detecting the expression of the protein.

Again, this method can work, but do not blindly rely on the compensation values from one experiment being able to correctly compensate samples in another experiment. Test antibody panels multiple times to see if compensation settings can be reused from one experiment to the next before relying on this method.

3.2 Dimming of Fluorochromes as a Result of Compensation

Compensation can result in a slight dimming of fluorochromes as a portion of the emission spectrum is subtracted during compensation. For this reason, it is recommended to use the brightest fluorochromes for low or moderately expressed antigens. This will help minimize any loss of signal.

3.3 Consider the Number of Overlapping Fluorochromes

Consider how many other fluorochromes spillover into your fluorochrome of choice. For example, PerCP, A647/APC, A700, and A750 all share a portion of the same emission spectrum and compensation, to some degree, will have to be performed across all combinations of those fluorochromes. This means that significant dimming, and in some case complete loss of signal, could occur. Test combinations of fluorochromes in pilot experiments to determine which combinations work well together before committing to a larger experiment.

3.4 Consider If Compensation Is Necessary

Can fluorochromes that have minimal spectral overlap be used or does the configuration of your flow cytometer allow for the use of multiple fluorochromes without compensation? For example, due to the configuration of BD LSRFortessa™ cytometers with 488, 561, 640, and 405 lasers, experiments can be performed with FITC/A488, PE, APC/A647, and A405/Pacific Blue™ fluorochromes without needing to compensate between those four PMTs. In the event that a fifth color is needed, a fluorochrome such as A700 would be a better choice than PerCP or PerCP/Cy5.5 as A700 will only spillover into the APC/A647 PMT (2-color compensation) whereas PerCP or PerCP/Cy5.5 will spill over into both the APC/A647 PMT and the PE PMT (3-color compensation).

3.5 Consider If Fluorochromes with High Spectral Overlap Can Be Separated on Two Different Cell Types

Some experiments may contain multiple cell types but the analysis may be limited to single-cell type. In this scenario, compensation could be avoided by distributing fluorochromes with high spectral overlap across both the cells of interest and unwanted cell types. For example, in Fig. 5.11, we are determining the expression of CD31 (PECAM) on CD4⁺ T cells using a mixed PBMC population. CD4⁺ T cells will be identified using a CD3 PE antibody and a CD4 FITC antibody. Using a BD LSRFortessa™ with separate 488 nm blue and 561 nm yellow/green lasers, compensation does not need to be performed between PE and FITC. To examine CD31 expression on T cells, a CD31 A700 antibody is used. To exclude B cells from analysis, B cells will be stained with a CD19 APC antibody. From earlier examples it is clear that the emission spectrums for A700 and APC overlap and would generally need to be compensated. However, since B cells will be excluded from the analysis, the fluorescence spillover between these A700 and APC is not a concern. Figure 5.11a shows the samples prior to compensation. 35.6% of the T cells express both CD4 and CD31 (CD4⁺ T cells) and 22.0% of the T cells do not express CD4 but express CD31 (CD8⁺ T cells). The MFI of CD31 expression on the CD4⁺ T cells is 995 and the MFI of CD31 expression on the CD8⁺ T cells is 1682. In Fig. 5.11b the spillover between CD19 APC and CD31 A700 can be clearly observed based on the diagonal nature of the cell population. Once compensation has been performed and the spillover between APC and A700 has been corrected, the position of the CD19/CD31 population has shifted (Fig. 5.11d) such that only a fraction of B cells express CD31. However, if we compare how compensation affected the percent of CD31 expressing cells and the MFI of CD31 expression are compared on T cells (Fig. 5.11c) we find that both the percent of CD4⁺ and CD8⁺ T cells that express CD31, as well as the MFI of CD31 expression, are nearly equivalent between the

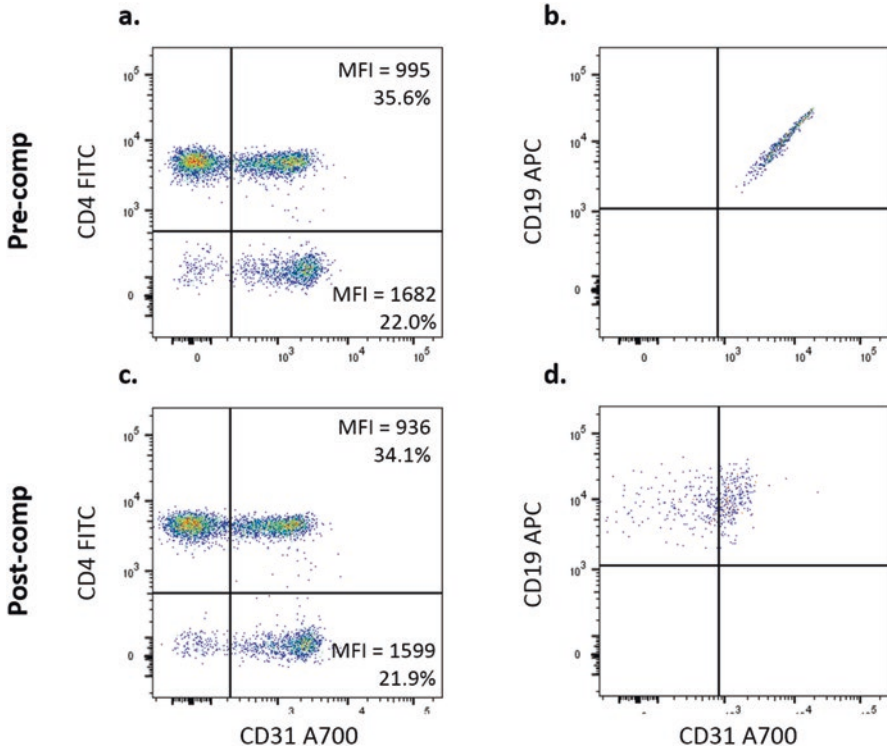


Fig. 5.11 Avoiding compensation by placing fluorochromes with overlapping spectral emission profiles on different cell types. Human PBMCs were stained with combinations CD3 PE, CD4 FITC, CD19 APC, and CD31 A700 antibodies. Plots show gated lymphocytes. (a) The MFI and percent expression of CD31 on CD3⁺CD4⁺ and CD3⁺CD4⁻T cells and (b) CD19⁺ B cells was determined before compensation was applied to correct for spillover between APC and A700. Notice the 45° angle indicating spillover between APC and A700 in the CD19 versus CD31 plot (b). Compensation was then applied and then (c) the MFI and percent expression of CD31 on CD3⁺CD4⁺ and CD3⁺CD4⁻T cells and (d) CD19⁺ B cells was again determined. The MFI and percent expression of CD31 on T cells did not change due to compensation despite the spillover between APC and A700 that needed to be corrected to analyze CD31 expression on B cells. Cells were stained with CD3 FITC and CD4 A700 antibodies. No CD19 APC antibody was included. The small percentage of spectral overlap of A700 into APC causes the CD4 A700 population to slightly drift toward the right (increased APC positive fluorescence). (b) Cells were stained with CD3 FITC and CD19 APC antibodies. No CD4 A700 antibody was included. The large percentage of spectral overlap of APC into A700 causes the CD19 APC population to significantly drift up (increased A700 positive fluorescence). (c) Cells were stained with all three antibodies and the effects of spillover of APC into A700 and A700 into APC can be seen on the same plot

same samples collected before compensation was performed (pre-comp) and after compensation was performed (post-comp). In other words, because the two fluorochromes that share fluorescence spillover (APC and A700) were on a cell type that was excluded from analysis (B cells), spectral overlap of the CD19 APC antibody into the CD31 A700 PMT did not affect the MFI of CD31 A700 detected on T cells.

3.6 *Using Cells as Single-Stain Controls vs. Beads*

The example of compensation shown earlier used compensation beads to correct for spectral overlap but some investigators prefer to use cells from their experiments to perform compensation; this is a perfectly valid method. One advantage of using cells as single-stain controls is that the cells used for compensation are of the same size and activation state as the cells being examined during the experiment. The disadvantage of using cells is that it might not always be possible to use the same antibody for compensation that you are using in your antibody staining panel. Whenever possible you should use the same antibody for compensation that you use in your staining panel as spillover values are unique to each antibody. Where this becomes a problem is when using an antibody specific for a protein that is only weakly expressed on cells. To perform accurate compensation, a clear positive and clear negative signal for each fluorochrome must be established. By their nature, compensation beads will provide a clear positive and clear negative population using virtually any antibody. This allows you to use the same antibody for compensation that is used in your antibody staining panel. If a protein is only weakly expressed on cells, there may not be a sufficiently clear positive and negative population to perform compensation. To circumvent this issue, a surrogate marker that is highly expressed on the cell of interest can be used for compensation. The rules are that (1) the fluorescence signal from the antibody used for compensation must be at least as bright as the fluorescence signal from the antibody used to stain your cells and (2) the same antibody must be used for all colors represented in your staining panel. An example of this is using an antibody against CD3 to perform compensation on T cells. CD3 is among the highest expressed proteins on T cells so staining T cells with an anti-CD3 antibody would provide for a sufficiently bright signal. Furthermore, when using a mixed population of lymphocytes (T cells, B cell, NK cells), the T cells would provide clear positive signal while the B and NK cells would provide a clear negative signal. When using an 8-color antibody panel to phenotype T cells, single-stain compensation controls could be created by staining T cells with an anti-CD3 antibody conjugated to all eight colors represented in the antibody panel. This would satisfy both rules for using cells as single-stain compensation controls.

4 Materials

4.1 *Flow Antibodies*

- hCD3 FITC (FAB100F; R&D Systems)
- hCD4 A700 (FAB3791N; R&D Systems)
- hCD4 FITC (FAB3791F; R&D Systems)
- hCD19 APC (FAB4867A; R&D Systems)
- CD31 A700 (FAB3567N; R&D Systems)

4.2 Staining Buffers/Fc Block Reagents

- Human IgG (1-001-A; R&D Systems)
- Flow cytometry staining buffer (FC001; R&D Systems)
- Positive Control anti-Mouse Ig, k Compensation Beads (BD)
- Negative Control Compensation Beads (BD)
- Flow Cytometry Human Lyse Buffer (FC002; R&D Systems)

References

1. Murphy K. Janeway's immunobiology. In: Garland science. 8th ed. New York: Taylor & Francis Group, LLC; 2012.
2. Compensation; an informal perspective. Mario Roederer. <http://www.drmr.com/compensation/index.html>

Chapter 6

Primary and Secondary Antibodies and Flow Cytometry Controls



Christopher Hammerbeck, Christine Goetz, and Jody Bonnevier

1 Antibody Development for Flow Cytometry

In flow cytometry, it is critical to use antibodies that are specific and sensitive. Antibodies for flow cytometry need to be able to detect proteins in a native conformation, often on a live cell. This is in contrast to antibodies used in Western blot, which require detection of a denatured, linearized protein. Monoclonal antibodies are generated by first immunizing animals with antigens. While peptide antigens may be used as immunogens, we have found that immunizing with full length, recombinant proteins has often skewed antibody production toward those that can detect natural protein epitopes on cells.

Following antibody generation, there is a critical need for any antibodies used in flow cytometry experiments to be rigorously validated and characterized, and specificity on cells verified. Antibodies are naturally sticky, more so on live cells, so validation is a critical step for antibody developers and manufacturers to ensure antibody specificity, rather than nonspecific stickiness. It is also critically important for users of flow cytometry antibodies to understand the characterization of the antibodies, how they were developed and manufactured, and to perform their own initial verification experiments to ensure that that antibody is performing as expected.

A rigorous antibody development and validation pipeline, such as the one at R&D Systems, ensures that only the highest-quality antibodies are available on market (Fig. 6.1). This is done by controlling the entire process, starting from antigen and antibody design, where input from customers, collaborators, and literature is incorporated into the antigen design. At early stages in development, antibodies are tested by direct ELISA and Western blot to determine that they bind their immu-

C. Hammerbeck · C. Goetz (✉) · J. Bonnevier

Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA

e-mail: Chris.Hammerbeck@bio-techne.com; Christine.Goetz@bio-techne.com; Jody.Bonnevier@bio-techne.com

© Springer Nature Switzerland AG 2018

C. Goetz et al., *Flow Cytometry Basics for the Non-Expert*, Techniques in Life Science and Biomedicine for the Non-Expert,
https://doi.org/10.1007/978-3-319-98071-3_6

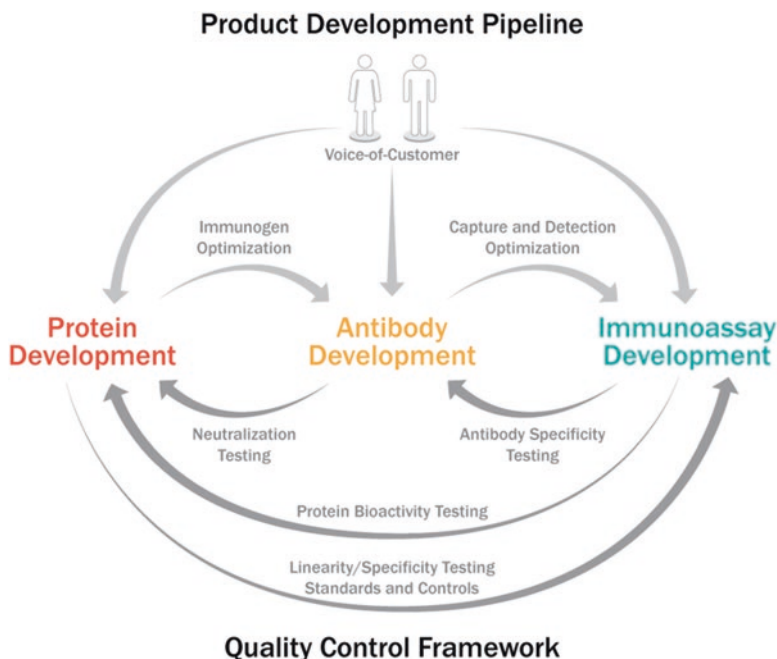


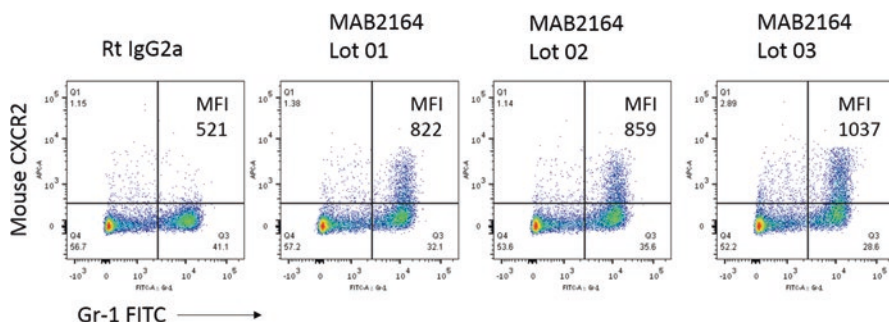
Fig. 6.1 Antibody development pipeline and quality control framework at R&D Systems

nogen. These techniques are also used to establish specificity, by testing to ensure that they do not bind to similar proteins, other protein family members, or other species, unless that is a desired characteristic of the antibody design.

Cell models are then designed to test the antibodies by flow cytometry. These models include positive and negative cell types such as cell lines, biological models like resting vs activated cells, costain phenotyping for expression on particular cell types within a mixed population, transfected cells overexpressing the protein of interest, or knock-out/knockdown cells lacking the protein of interest. Simultaneously, antibodies are tested in other applications, including Western blot(WB), Immunocytochemistry (ICC), Immunohistochemistry (IHC), and ELISA matched pairs, in addition to flow cytometry. This rigorous validation and characterization generates antibodies that are well suited for flow cytometry experiments.

Those clones picked during the development phase to become flow cytometry products are then tested again in large scale production, and again after bottling before release to customers. In addition, every new production lot of antibody is tested side-by-side with the previous lot to ensure reproducibility (Fig. 6.2). These stringent quality control parameters help ensure the highest-quality antibody is released to customers.

The antibody development strategy outlined above is very similar to the process used by the Human Cell Differentiation Molecules (HCDM) group, who is in charge of evaluating antibodies and defining new Cluster of Differentiation (CD) markers



Three lot comparison of rat anti-mouse CXCR2

Cells: B6 mouse bone marrow

Stain: surface; Gr-1 co-stain

Fig. 6.2 Quality control to ensure reproducibility. Mouse bone marrow cells were stained with Rat anti-mouse CXCR2 monoclonal antibody or Rat IgG2A isotype control antibody. Side-by-side data is shown for flow staining of three different lots of antibody

at the Human Leukocyte Differentiation Antigen (HLDA) Workshops (<http://www.hcdm.org/>). These are blinded and comprehensive, independent, validations, and antibodies must meet stringent criteria, including specific staining on positive, but not negative cell types, including transfectants and natural cell subsets. In addition, at least two independent antibodies must have identical staining patterns to receive the new CD markers designation. In the most recent HLDA workshop, an R&D Systems clone received the HLDA designation for each of the newly named CD markers defined in this conference, highlighting the importance and relevance of the R&D Systems in-house antibody development and validation process for flow cytometry antibodies (Fig. 6.3).

Why is this so important to your flow cytometry experiments? Concerns about improperly characterized antibodies used in biomedical research have been raised and reproducibility issues have resulted in loss of time and resources [1]. Five antibody validation pillars have been outlined by the International Working Group for Antibody Validation [2].

These include:

1. Genetic strategy: Target protein is reduced or eliminated by genome editing.
2. Orthogonal strategy: Target protein expression compares with an antibody-independent method of detection.
3. Independent antibody strategy: Target protein expression is compared with two independent antibodies with nonoverlapping epitopes across a panel of samples.
4. Expression of tagged proteins: Correlate target protein signal with antibody and a tag-specific signal.
5. Immunocapture mass spectrometry: Detection of protein abundance after capture and mass spectrometry.

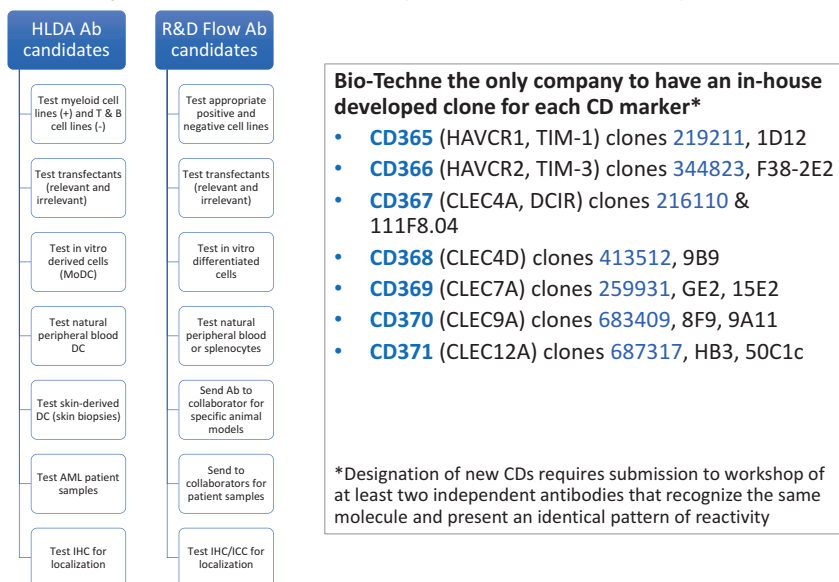
HLDA Workshop 10: Focus on Dendritic Cells (New Molecules CD 365-371)

Fig. 6.3 HLDA10 validation process compared to R&D Systems flow antibody validation process. The R&D Systems antibody validation process is similar to that used in the HLDA10 validation workshop. An R&D Systems monoclonal antibody received the HLDA designation for each of the newly named CD markers

As an example, genetic strategies have been utilized for enhanced specificity testing, including CRISPR knockouts. In Fig. 6.4, Ki-67/MKI67 is specifically detected by immunocytochemistry in HeLa cells, but not in Ki-67/MKI67 knockout HeLa cells, using a Human Ki-67/MKI67 Monoclonal Antibody. AXL is also specifically detected by flow cytometry in A431 cells, but not in AXL knockout A431 cells, using a Human Axl Monoclonal Antibody. By using these knockout validated antibodies, researchers can be more confident in their experimental results. Although direct fluorochrome-conjugated antibodies are desirable, they may not always be available in the antibody-fluorochrome combination desired, and so in the next section, we'll discuss how to use unconjugated antibodies in flow cytometry.

2 Detection of Unconjugated Primary Antibodies

Most of the flow cytometry antibodies that we use today are directly conjugated to a fluorochrome. In other words, an antibody has been chemically linked to a fluorochrome. The addition of the fluorochrome is necessary for the antibody to be seen by the flow cytometer. As we discussed in Chap. 2, when a fluorochrome is excited by the flow cytometer's excitation laser, the fluorochrome emits light. The light is

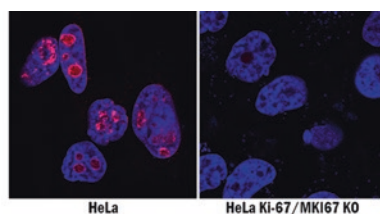
Validation Pillar: Genetic Strategies

CRISPR knockout validation initiative for specificity testing

Knockout Validated

MAB7617

Ki-67/MKI67 Specificity shown by
ICC in KO Cell Line (HeLa)



Knockout Validated

MAB154

AXL Specificity shown by Flow in
KO Cell Line (A431)

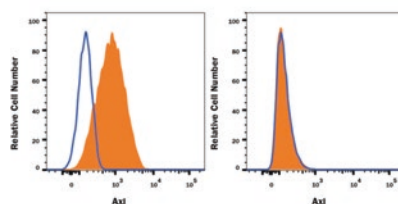


Fig. 6.4 Antibody validation by CRISPR knockout. Genetic strategies, such as CRISPR knockouts, are an important antibody specificity tool. Wild-type HeLa cells and Ki-67 knockout HeLa cells were stained with anti-Ki-67 antibody, and A431 and AXL-knockout A431 cells were stained with anti-AXL antibody, showing antibody specificity

then detected and amplified by the cytometer's PMT and converted to a digital signal that is displayed on a flow plot. If an antibody is not coupled to a fluorochrome, no light will be emitted and detected by the PMT. In a perfect world, all the antibodies we use would be directly conjugated; however, in some cases it is necessary to use unconjugated antibodies, or antibodies that are not directly attached to a fluorochrome. In these circumstances, we need to provide a separate fluorescent signal that can be detected by the cytometer's PMT. This separate signal is provided by a directly-conjugated secondary antibody which binds directly to the primary antibody.

The secondary antibody displays affinity for the isotype and species of the unconjugated primary antibody that you are using. Just like a primary antibody has specificity for, and binds to, a particular protein (e.g., human CD4, mouse CD19, rat CD3), a secondary antibody has specificity for, and binds to, a portion of the primary antibody. Without a directly-conjugated secondary antibody, the unconjugated antibody would be invisible to the cytometer.

Primary and secondary antibodies have many similarities but also have some noticeable differences. While most primary antibodies are made in smaller rodents (e.g., mice and rats), most secondary antibodies are made in larger species (e.g., goats, sheep, donkeys). This is partly due to the conservation of immunoglobulin (i.e., antibody) genes between species and the ability of the immune systems in closely related species to mount an immune response against proteins that are seen as self-proteins. For example, mice and rats share a greater degree of genetic homol-

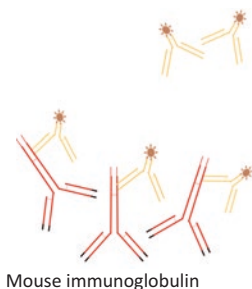
ogy in their immunoglobulin genes than mice and goats. The immune system in a rat is therefore more likely to see a mouse immunoglobulin as a self, or mouse, immunoglobulin resulting in a very weak immune response. By contrast, the immune system in a goat is more likely to see a mouse immunoglobulin as a foreign immunoglobulin resulting in a strong immune response. The strong immune response in the goat will likely produce many goat antibodies that bind to mouse antibodies. In this example, the antibodies produced would be called goat (because they were made in a goat) anti-mouse IgG antibody (because the immunogen was a mouse IgG). If the process had been completed in a donkey, the antibodies produced would be called donkey anti-mouse IgG antibody (because the antibodies were made in a donkey and the immunogen was a mouse IgG). In other words, secondary antibodies are defined by their host species (the animal in which they are made) and their target species reactivity (the species of antibody and isotype to which they bind). The host for a goat anti-mouse IgG secondary antibody would be a goat and the reactivity would be all mouse IgG. The host for a rabbit anti-mouse IgG2a secondary antibody would be a rabbit and the reactivity would be only mouse immunoglobulin with a IgG2a isotype. Most secondary antibodies used in flow cytometry are made in either donkeys or goats and are polyclonal antibodies, but some are also made in rabbits, rats, and mice. Unless specifically designed to bind a specific isotype, most secondary antibodies will bind any IgG from a given species be it IgG1, IgG2a, IgG2b, IgM, and so forth. A list of common secondary antibodies is found in Table 6.1.

Primary antibodies are also typically monoclonal antibodies while secondary antibodies are typically polyclonal antibodies. As a secondary detection reagent, polyclonal antibodies have the advantage of being able to amplify the signal of the primary antibody. Remember that a monoclonal antibody is an antibody or group of antibodies that has specificity for a single epitope on a given protein. In comparison,

Table 6.1 Common secondary antibodies

Host species	Target species	Reactivity	Common name
Donkey	Mouse	IgG	Donkey anti-mouse IgG
	Goat	IgG	Donkey anti-goat IgG
	Rabbit	IgG	Donkey anti-rabbit IgG
	Sheep	IgG	Donkey anti-sheep IgG
Goat	Rabbit	IgG	Goat anti-rabbit IgG
	Mouse	IgG	Goat anti-mouse IgG
	Mouse	IgM	Goat anti-mouse IgM
	Rat	IgG	Goat anti-rat IgG
	Chicken	IgY	Goat anti-chicken IgY
	Human	IgG	Goat anti-human IgG
Rabbit	Mouse	IgG2a	Rabbit anti-mouse IgG2a
Rat	Mouse	IgG2a	Rat anti-mouse IgG2a
	Mouse	IgG2b	Rat anti-mouse IgG2b
Mouse	Hamster	IgG	Mouse anti-hamster IgG

Monoclonal Goat-anti mouse IgG 2° antibody

a.

Polyclonal Goat-anti mouse IgG 2° antibody

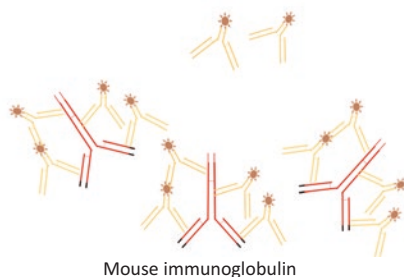
b.

Fig. 6.5 Fluorescence amplification using polyclonal secondary conjugated antibodies. **(a)** Monoclonal goat anti-mouse secondary conjugated antibodies (yellow) bind to only a single epitope on mouse IgG. The restriction to bind a single epitope limits the number of conjugated secondary antibodies that can bind to the mouse IgG molecule. **(b)** Polyclonal goat anti-mouse secondary conjugated antibodies (yellow) bind to multiple epitopes on the mouse IgG molecule. The fluorescence of the conjugated secondary antibody is amplified as more mouse IgG epitopes are bound by the conjugated secondary antibody

a polyclonal antibody is a group of antibodies that have specificity for multiple epitopes on the same protein. If we extend this to our goat anti-mouse IgG antibody example in Fig. 6.5, a monoclonal goat anti-mouse IgG antibody would only bind to one epitope on all mouse immunoglobulin Fig. 6.5a whereas a polyclonal goat anti-mouse IgG antibody would bind to multiple epitopes on all mouse immunoglobulin Fig. 6.5b, essentially multiplying the fluorescence signal by virtue of there being more fluorochrome-conjugated goat secondary antibodies bound to the primary mouse antibody.

So how do we put this together in a practical way? In Fig. 6.6a we have an unconjugated antibody specific for human CD3 that was made in a mouse and is represented by the red antibody. We would also call this a mouse anti-human CD3 antibody. On the right we have an unconjugated antibody specific for human CD3 that was made in a rat and is represented by the blue antibody. We would also call this a rat anti-human CD3 antibody. Our secondary antibody, represented in yellow, is specific for mouse IgG and was made in a goat. We would call this a goat anti-mouse IgG secondary antibody. As shown in Fig. 6.6b, the goat anti-mouse IgG secondary antibody will not bind IgG that are from rats but will bind any IgG that are from mice. Similarly, a goat anti-rat IgG secondary antibody will not bind IgG that are from mice but will bind any IgG that are from rats.

Since most secondary antibodies will bind to any antibody made in a given species, when using unconjugated antibodies with secondary antibodies, it is necessary that we add all our flow antibodies in a particular order, especially when using multiple antibodies from the same species. Consider the following example. We want to detect CD19 expressed on human B cells. Our CD19 antibody is made in mice but is unconjugated and will therefore require us to use a goat anti-mouse secondary

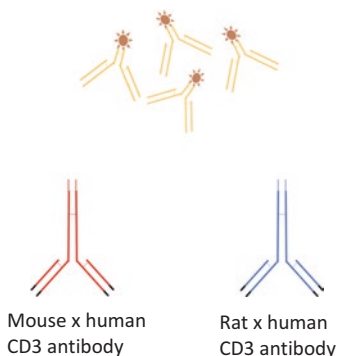
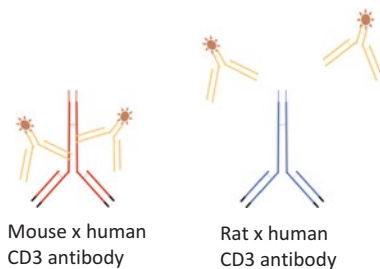
a. Goat-anti mouse IgG 2° antibody**b.** Goat-anti mouse IgG 2° antibody

Fig. 6.6 How do unconjugated and secondary antibodies work? **(a)** The red antibody represents an antibody that is made in mice and is specific for human CD3 (mouse anti-human CD3). The blue antibody represents an antibody that is made in rats and is specific for human CD3 (rat anti-human CD3). The yellow antibody represents a secondary antibody that is made in goats and is specific for all mouse IgG antibodies (goat anti-mouse IgG). **(b)** When used together, the goat-anti-mouse IgG secondary antibody binds only to the mouse anti-human CD3 antibody and not to the rat anti-human CD3 antibody

antibody. We want to make sure that our CD19 antibody binds to B cells only. To check this specificity, we will use a CD3 antibody that is also made in mice to identify T cells. CD19 should not show up on our T cells. This means we have two antibodies that are made in mice; a goat anti-mouse secondary antibody will recognize both the CD19 and CD3 antibodies. Because of this, we need to add our antibodies in a particular order. Figure 6.7a outlines the correct order. First, we would add the mouse anti-human CD19 antibody. After a short incubation, any excess CD19 antibody would be washed away and a goat anti-mouse PE secondary antibody would be added. After another short incubation, any excess secondary antibody would be washed away and then a mouse anti-human CD3 FITC antibody (costain) would be added last. When the staining process is performed in this order you end up with the appropriate staining pattern indicated by Fig. 6.7b. Three distinct populations are visible: A CD3 positive population (T cells), a CD19 population (B cells), and a third population that is neither CD3 nor CD19 positive (NK cells). Because the antibodies were added in the correct order, none of the T cells appear to be CD19 positive. The goat-anti mouse secondary antibody was washed away before the mouse anti-human CD3 antibody was added. Because the excess secondary antibody was washed away it was not able to bind to our mouse-anti human CD3 antibody. The only antibody it could bind was the mouse anti-human CD19 antibody (Fig. 6.7c).

Figure 6.8a outlines the incorrect staining order. In this example, the unconjugated mouse anti-human CD19 and mouse anti-human CD3 antibodies were added together. After a short incubation these antibodies were washed away, and a goat

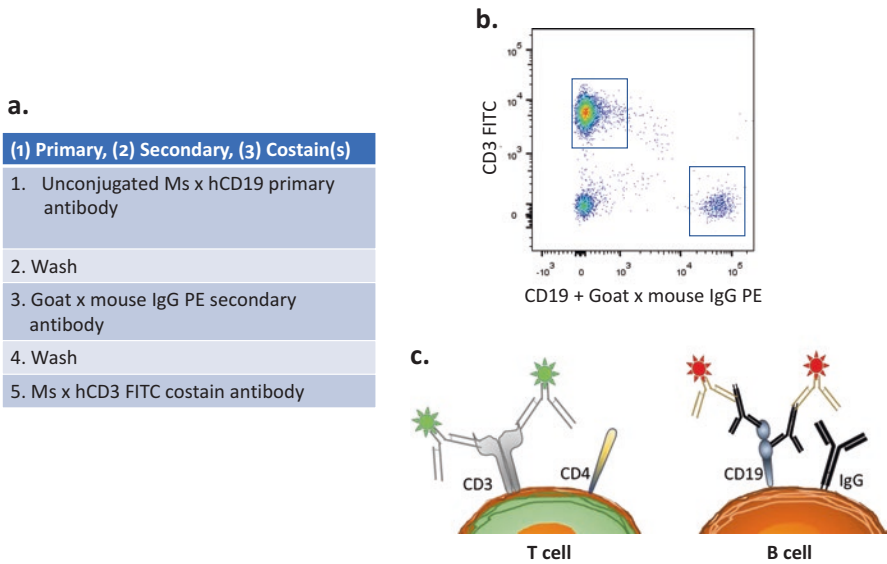


Fig. 6.7 Using unconjugated and secondary antibodies in the correct order. A mouse anti-human CD3 FITC antibody, an unconjugated mouse anti-human CD19 antibody and a goat anti-mouse PE secondary antibody were used to detect T and B cells in human PBMCs. **(a)** The unconjugated CD19 antibody was added first, the goat anti-mouse secondary antibody was added second and the CD3 costain was added last. **(b)** Staining in the correct order results in three distinct cell populations. The CD3⁺ T cells do not appear to express CD19 since the CD3 costain was added after the secondary antibody was washed away. Thus, it was not bound by the goat anti-mouse secondary antibody **(c)**

anti-mouse PE secondary antibody was added. The result (Fig. 6.8b) is a mess. Most of the cells appear to be both CD3 and CD19 positive and the T cells appear to express CD19 so highly that the population is nearly off-scale. In this situation, both the mouse anti-human CD19 and mouse anti-human CD3 antibodies were added at the same time. Consequently, the goat-anti mouse secondary antibody recognized both antibodies as mouse and bound to both the CD19 and CD3 antibodies (Fig. 6.8c). The result is that both the T cells and B cells will appear to be PE-positive and because both cell subsets are labeled with the goat anti-mouse PE secondary antibody the PE fluorescence is more than doubled, moving the PE-positive population further to the right.

3 Flow Cytometry Controls

Flow cytometrists have a love/hate relationship with isotype control antibodies and have strong opinions about which types of controls should be used in the context of a flow cytometry experiment. Here, we hope to take a measured approach to this

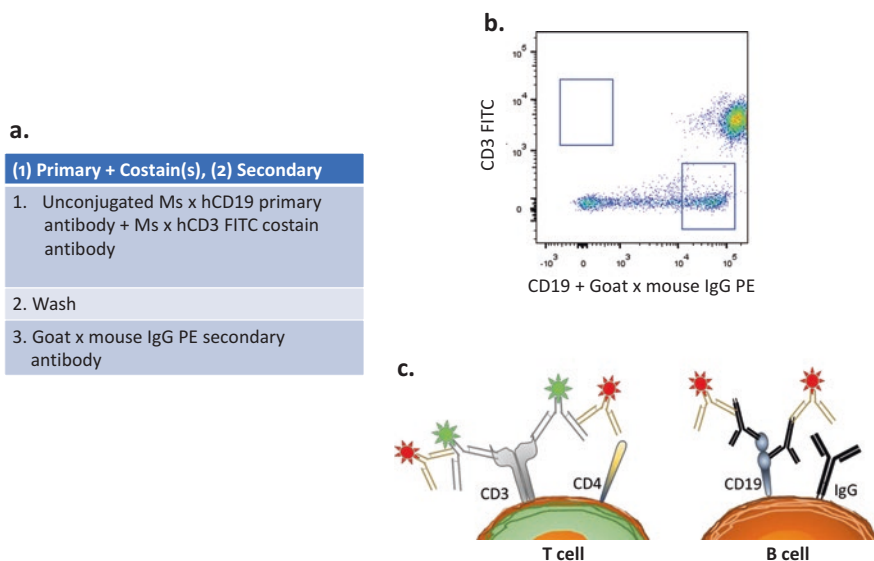


Fig. 6.8 Using unconjugated and secondary antibodies in the incorrect order. A mouse anti-human CD3 FITC antibody, an unconjugated mouse anti-human CD19 antibody and a goat anti-mouse PE secondary antibody were used to detect T and B cells in human PBMCs. **(a)** The unconjugated CD19 antibody and mouse anti-human CD3 FITC antibody were added first and the goat anti-mouse secondary antibody was added last. **(b)** Staining in the incorrect order results in inappropriate placement of the cell populations. The CD3⁺ T cells appear to express CD19 since the CD3 costain was added before the secondary antibody was washed away. Thus, it, along with the CD19 antibody were both bound by the goat anti-mouse secondary antibody **(c)**

topic and discuss how isotype control antibodies can be used along with other types of controls such as fluorescent controls, lineage controls, and cell activation controls to properly set up and analyze flow cytometry experiments.

3.1 Isotype Controls

There are five main classes of antibodies in mice and humans: IgA, IgD, IgE, IgG, and IgM. The IgG class is the most common class of antibody induced during an immune response to a pathogen or foreign protein, such as would be the case when immunizing mice to develop antibodies that are specific for human proteins. The IgG class can further be broken down into four main subtypes: IgG1, IgG2a, IgG2b, and IgG3. The first three of these (IgG1, IgG2a, and IgG2b) represent the most common class and isotypes of commercially available antibodies and are represented by the antibodies with the blue, purple, and yellow Fc heavy chains, respectively (Fig. 6.9).

Just like you would include controls for your experiments, one purpose of isotype control antibodies is to act as controls in the process of manufacturing antibody-

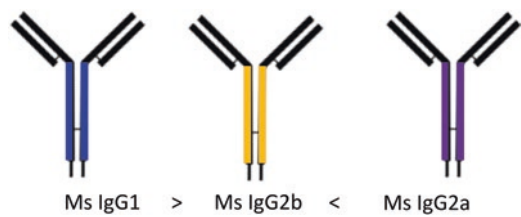


Fig. 6.9 Common Mouse Immunoglobulin isotypes used for flow cytometry antibodies. The three most common mouse IgG isotypes used for flow cytometry antibodies are shown here. Ms IgG1 (blue) is the most common isotype generated following immunization of mice with non-mouse proteins followed by Ms IgG2a (purple) and then Ms IgG2b (yellow)

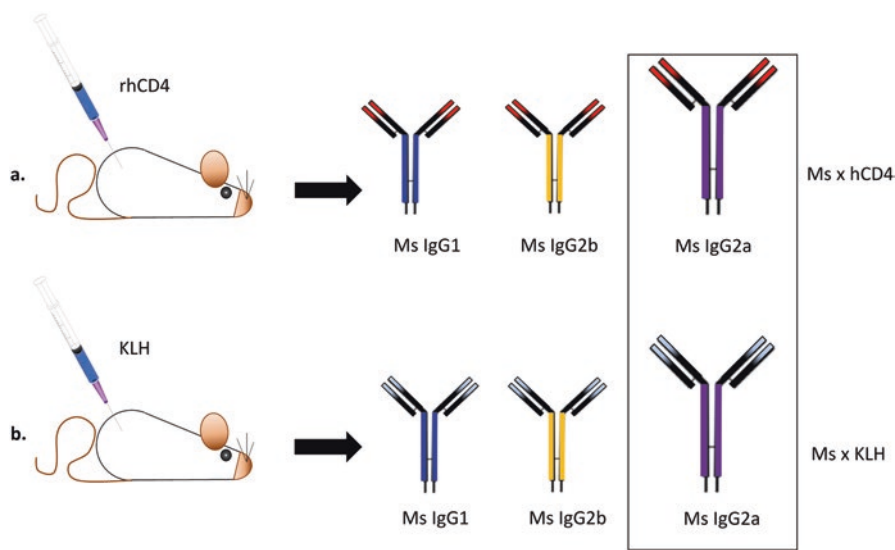


Fig. 6.10 Theoretical generation of antibodies in mice. Mice are immunized with recombinant human CD4 protein (a) or control KLH protein (b). Immunization results with antibodies of all three isotypes, IgG1, IgG2a, IgG2b. The anti-CD4 antibody selected is of the Ms IgG2a background (a) therefore a Ms IgG2a antibody generated upon immunization with the KLH protein (b) is selected as the appropriate isotype control

ies. For example, if we are creating an antibody in mice that is specific for human CD4, one group of mice would be immunized with the human CD4 protein while another “control” group of mice would be immunized with an irrelevant “control” protein that is immunogenic but is not found in either mice or humans. Oftentimes, the KLH antigen is used as this control protein. KLH is a protein antigen found in the giant keyhole limpet, an aquatic organism, and it is not found in mice or humans.

An example of how this process works can be found in Fig. 6.10. Here, immunization of mice with recombinant human CD4 protein (Fig. 6.10a) resulted in an antibody that is specific for human CD4 (represented by the red binding domain on

the antibody) and that possesses an IgG2a isotype (represented by the purple Fc heavy chain). To create an isotype control antibody for our CD4 antibody (Fig. 6.10b), we would then select an antibody with an IgG2a isotype from mice immunized with the KLH control protein (the antibody represented by the light blue binding domain and the purple Fc heavy chain). Therefore, the isotype control antibody for this example is an antibody that lacks specificity to the CD4 target but matches the class (IgG) and type (2a) of the primary CD4 antibody. In order to be useful, the isotype control antibody should also have the same fluorochrome as well as the same fluorescence to protein (F:P) ratio as the primary antibody. The F:P ratio is a ratio of the number of fluorescence particles attached to an antibody. If the isotype control antibody has a higher or lower F:P ratio than the corresponding primary antibody, the background fluorescence of the isotype control antibody will accordingly be higher or lower than the primary antibody in which case it will be difficult to accurately predict and control for the background fluorescence of your primary antibody. In this way isotype control antibodies control for both the antibody manufacturing and conjugation process and should, ideally, be obtained from the same vendor as your primary antibodies.

Given their similarity to primary antibodies, isotype controls can be used as negative controls to help distinguish any nonspecific background signals from specific antibody signals. Isotype controls are useful for testing panels of antibodies and antibodies that are newly acquired in the lab for specificity. Causes of nonspecific background signals and staining can include binding to Fc receptors on target cells, nonspecific antibody interactions with cellular proteins, carbohydrates, and lipids, antibodies nonspecifically trapped during intracellular staining, and autofluorescence of target cells. It should be noted; however, that isotype controls cannot address the cross reactivity of a primary antibody for related epitopes on different antigens nor can they control for reactivity with different epitopes (binding sites) within the same antigen.

In Fig. 6.10 we discussed the generation of a theoretical mouse anti-human CD4 antibody and corresponding Mouse IgG2a isotype control antibody. In the dot plots in Fig. 6.11 we show human lymphocytes stained with an actual APC-conjugated CD4 antibody (Fig. 6.11a) or corresponding APC-conjugated Mouse IgG2a isotype control antibody (Fig. 6.11b). When we stain lymphocytes with our Mouse IgG2a isotype control antibody (Fig. 6.11b) we see that we only have two populations of cells—a CD3 positive and CD3 negative population. Both populations show no APC fluorescence along the X-axis. Since CD4 is the primary antibody being used in this experiment, we would then consider these populations to be our CD4 negative cells. We would adjust the voltage of the APC channel to generally place these negative populations between 0 and 10^2 , a.k.a. the first two decades, on the log scale. It's worth noting that when adjusting the PMT voltage, or gain for each channel, the same strategy cannot always be used for every fluorochrome. Gain can be defined as an adjustment made to increase or lower the PMT voltage by regulating the sensitivity of the PMT. Small changes to the PMT voltages for fluorochromes with shorter wavelengths such as A405, FITC, and PE will result in fairly substantial shifts in the placement of cells along the axis whereas fluorochromes with lon-

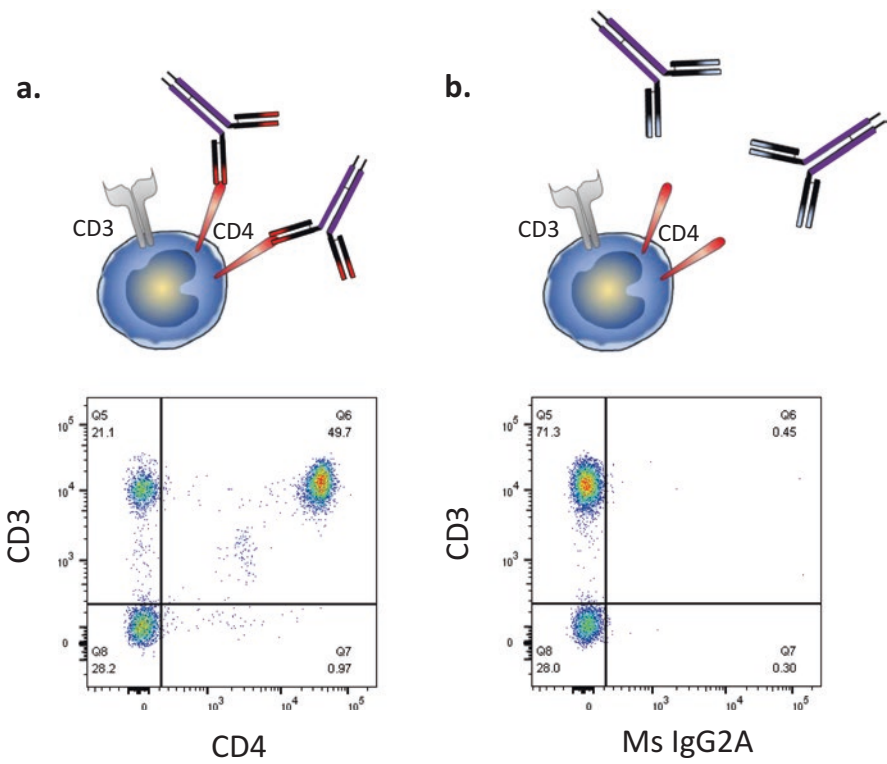


Fig. 6.11 Flow cytometry staining with antigen-specific versus isotype control antibodies. Mouse antibodies specific for human CD4 bind to the CD4 protein on CD3⁺ T cells (a) while isotype control antibodies generated in mice immunized with the KLH protein do not bind (b). When human PBMCs are stained with the antigen-specific mouse anti-human CD4 antibody, a positive fluorescence signal is detected while no positive signal is detected when using the corresponding mouse IgG2a isotype control antibody

ger wavelengths such as A647, A700, and A750 may exhibit little to no shift no matter how much the voltage is adjusted. This is due to the propensity of cells to autofluoresce more at shorter wavelengths than at longer wavelengths. Therefore, increasing the gain settings in the shorter wavelength channels amplifies the autofluorescence signals more so than increasing the gain settings in the longer wavelength channels. The goal is to select a voltage that is high enough to clearly resolve a positive signal from background noise but one that is low enough so that all positive signals are on scale and that compensation is not severely affected. (Refer to Chap. 5 where we discussed how changes to A700 and APC voltages impacted the compensation values for the two fluorochromes).

Once we have established the APC voltage that positioned our cells within the first two decades, we would then draw our APC gate just to the right of our APC negative cells. In this way we are setting our gate on the negative staining pattern of our isotype control antibody. The CD3 FITC gate would be drawn between the CD3

positive and CD3 negative populations. In the dot plot to the right we see that when we stain our cells with the APC-conjugated CD4 antibody our CD4 positive population now shows up as a separate distinct population in the upper right quadrant. Because our isotype control staining shows us any nonspecific background staining that we could expect from our CD4 antibody, we can be confident that the population of CD3 positive cells that show up in the upper right quadrant truly express CD4. There are many mixed opinions on using isotype control antibodies to establish positive and negative gates. Multiple controls are available, and some are more appropriate than others for a given experiment. The example we have just provided is a very simple example of gating based on isotype control antibodies. In reality, establishing positive and negative gates for bimodal populations, such as CD3 versus CD19 or CD3 versus CD4 in the example above would be best accomplished using a different type of biological control that we will discuss next.

In summary, an isotype control antibody therefore is a control antibody that lacks specificity to a target antigen but matches six important characteristics of the antigen-specific primary antibody:

1. Fluorochrome (PE, APC, etc.).
2. Species (mouse, rat, hamster, etc.).
3. Heavy chain class (IgA, IgG, IgD, IgE, or IgM).
4. Light chain class (kappa or lambda).
5. Type (1, 2a, 2b, 3, etc.).
6. F:P ratio.

3.2 *Internal Lineage Controls*

A second type of control that we might use to establish positive and negative signals is a type of biological control involving the use of lineage markers. This type of control takes advantage of cells that are known to not express a particular protein and will thus stain “negative” when using an antibody specific for that protein. You would also describe this as a bimodal distribution existing within a population of cells. For example, in human blood we can find three distinct populations. The smallest in terms of side and forward light scatter is the lymphocyte population. Above and to the right of that is the monocyte population and above the monocytes are a large population of granulocytes (Fig. 6.12a). As an example of how to use lineage markers as control proteins, let’s compare CD14, CD19, and CD3.

Lymphocytes can be broken down into three major, distinct populations: T cells that uniquely express CD3, B cells that uniquely express CD19, and NK cells that express neither CD3 nor CD19 but uniquely express CD56. CD3 is the lineage marker for T cells, CD19 is a lineage marker for B cells and CD56 is the lineage marker for NK cells. When we stain lymphocytes with CD19 and CD3 antibodies (Fig. 6.12b) we find three distinct cell populations: a CD19⁺/CD3⁻ B cell population in the upper left quadrant, a CD3⁺/CD19⁻ T cell population in the lower right

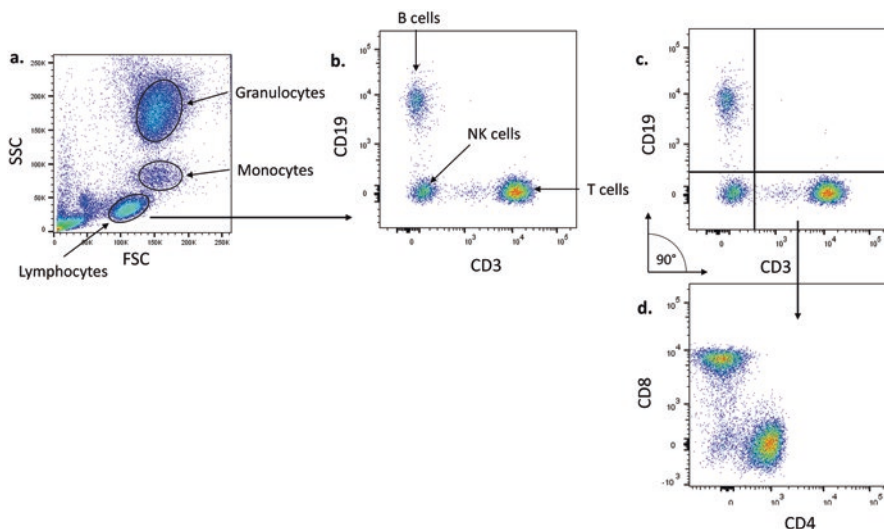


Fig. 6.12 Establishing positive and negative gates using multiple internal lineage marker controls. Human PBMC can be separated into three main populations: lymphocytes, monocytes, and granulocytes (a). Lymphocytes (b) can be divided into three main populations (B cells, NK cells, and T cells) based on the expression of the lineage markers CD19 and CD3. Negative gates for both the X- and Y-axis are established just outside of the CD3/CD19 double negative population (c). CD3⁺ T cells can further be broken down into two subsets (CD4⁺ and CD8⁺) based on the expression of the lineage markers CD4 and CD8 on T cells (d)

quadrant, and a CD3⁻ / CD19⁻ NK cell population in the lower left quadrant. The B cells do not express CD3, so we can use the CD19 positive B cells as a negative internal or lineage control for CD3 expression. The T cells do not express CD19, so we can use the CD3 positive T cells as a negative internal or lineage control for CD19 expression. Similarly, the NK cells do not express either CD19 or CD3, so we can use NK cells as a negative internal or lineage control for both CD3 and CD19. The populations are bimodal along both the X- and Y-axis and because of this, we can set our negative gate slightly outside the double negative NK cell population (Fig. 6.12c). All three populations form a 90° angle. Additionally, as shown in Fig. 6.12d, within the peripheral T cell compartment we have distinct populations of T cells that express CD4 or CD8. Rarely do we find peripheral T cells expressing both CD4 and CD8, whereas in the thymus, double positive CD4⁺CD8⁺ T cells are common as this is a stage in T cell development. Therefore, we can use CD4 as internal or lineage control for CD8 expression and vice versa. Even within the thymus where CD4 and CD8 can be co-expressed on the same cell, there is usually a clear enough distinction between the CD4-single positive cells, the CD4- and CD8-double positive cells and the CD8-single positive cells that you can use the bimodal distribution of CD4 and CD8 to establish positive/negative gates without the use of an isotype control antibody.

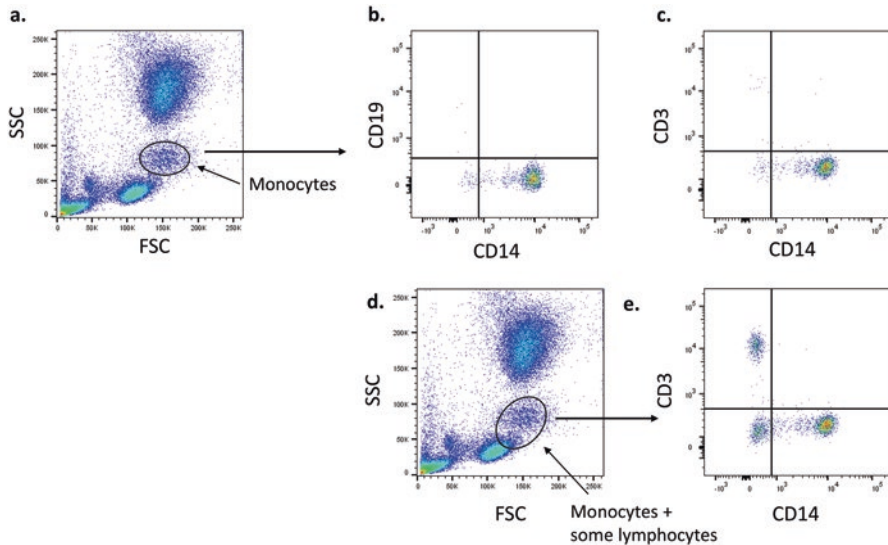


Fig. 6.13 Establishing positive and negative gates when only a single internal lineage marker control is present. Human monocytes (a) uniquely express CD14 and do not express CD19 (b) or CD3 (c). When gating only on monocytes, no substantial CD14 negative population exists making it difficult to establish where to set a negative gate. By expanding the monocyte gate to include a small percentage of lymphocytes (d) a CD14 negative population can be visualized (e) allowing for the correct placement of negative gates along the X- and Y-axis

Now let's look at the monocyte population (Fig. 6.13a). The monocyte population is only composed of one cell type, monocytes. Of our three example proteins, monocytes are known to uniquely express CD14; it is the lineage marker for monocytes. They do not express CD3 or CD19 as shown in Fig. 6.13b, c respectively. Knowing that monocytes are uniquely CD14⁺, how do we establish a positive/negative gate when we have a unimodal population? One option would be to use an isotype control antibody, but another easy method would be to temporarily adjust the size of the monocyte gate to include a small percentage of lymphocytes (Fig. 6.13d) and take advantage of the fact that lymphocytes do not express CD14. As shown in Fig. 6.13e, we can use the CD3 positive T cells as a negative internal or lineage control for CD14 expression since T cells do not express CD14 and set our negative gates just outside of the CD3⁺CD14⁻ double negative population. One word of caution when expanding the monocyte gate is to include a CD14 negative population. The monocyte gate should only be expanded in the lymphocyte population and not into the granulocyte population as granulocytes can also express a moderate level of CD14. Also keep in mind that a separate, minor, CD14 negative population of monocytes does exist and, if large enough, may be sufficient to establish a positive/negative gate without including lymphocytes.

While CD4 and CD8 can be used as lineage markers within the T cell compartment, outside of the T cell compartment, CD4 and CD8 are unreliable internal or

lineage controls because both proteins can be expressed by cells other than T cells such as monocytes and dendritic cells. For example, monocytes and macrophages express intermediate levels of CD4 which can change depending on the activation and differentiation status of the monocyte and macrophage. To illustrate the detection of CD4 on monocytes, in Fig. 6.14a we have expanded our live gate to include both lymphocytes and monocytes. We have color coded the populations within the expanded gate to make them easy to visually distinguish (Fig. 6.14b). The orange population is CD14⁺ monocytes, the blue population is CD4⁺ T cells, the purple population is CD8⁺ T cells, and the red population is composed of B cells and NK cells. In Fig. 6.14c we can see four distinct populations, the CD3⁻CD4⁻ double negative “red” B cell / NK cell population, a CD3⁺CD4⁻ “purple” CD8⁺ T cell population, a CD3⁺CD4⁺ double positive “blue” CD4⁺ T cell population, and an orange CD3⁻CD4^{lo} population. In Fig. 6.14d we can see that this orange population is CD14⁺ indicating these are monocytes. Here we have a tri-modal CD4 distribution; T cells have high CD4 expression, B cells and NK cells have low CD4 expression, and resting monocytes have intermediate CD4 expression. Since we have clear negative populations in Fig. 6.14c, d we can use this internal biological control to set our negative CD4 gate. If we compare the position of our negative gates using an isotype control antibody (Fig. 6.14e, f), we can see that the gates we set using the internal control populations agree with the negative staining pattern obtained using

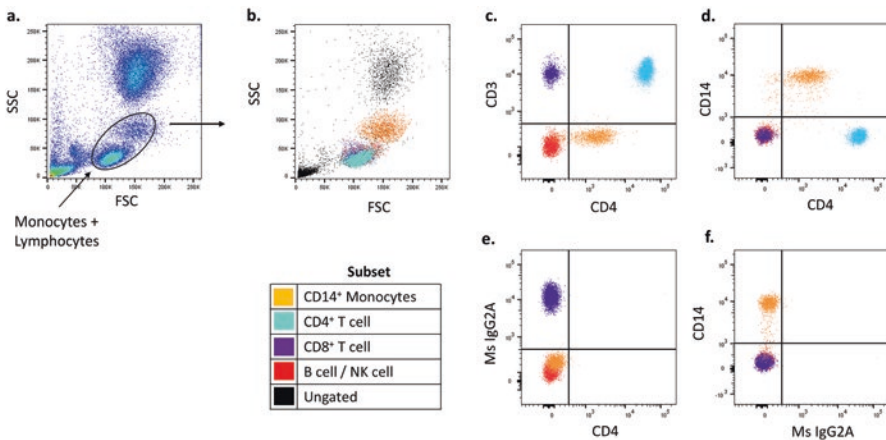


Fig. 6.14 Using a combination of isotype and internal lineage marker controls to establish negative CD4 gates for monocytes. Human whole blood was stained with hCD3 FITC, hCD14 APC, and hCD4 PE or Ms IgG2a PE antibodies. Lymphocytes and monocytes were gated (a) and a false color plot (b) generated representing each of the relevant populations: yellow = monocytes; blue = CD4⁺ T cells, purple = CD8⁺ T cells, red = B and NK cells; black = all remaining ungated cells. (c) CD3, CD4 and CD14 negative gates set by internal lineage marker controls (c and e). (c) Comparison of CD3 versus CD4 show a CD3⁻ cell type expressing intermediate levels of CD4 (yellow) which are found to be CD14⁺ monocytes (d). CD4 negative gates set by Ms IgG2a isotype control staining (d and f). Monocytes show no background staining using Ms IgG2a isotype control antibody (f)

a Mouse IgG2a isotype control antibody in place of a Mouse anti-human CD4 antibody. Certain mouse IgG isotypes can display different background staining on cells and Mouse IgG2a antibodies can be particularly messy when staining monocytes and macrophages so blocking Fc receptors is a must. In this scenario, using an isotype control antibody to confirm staining pattern found when using an internal biological control to set a gate can be important in that the isotype control antibody controls for any nonspecific antibody “stickiness” which might be missed using an internal control population alone.

As a reminder, the use of internal or lineage controls must take into account the protein of interest and the cell type you are using.

3.3 Cell Activation Controls

We’ve discussed so far when to use isotype controls and when it would be ok to use internal lineage controls instead. Now let’s build on that and discuss the use of isotype controls and FMO controls in the context of cell activation markers. Consider the example presented in Fig. 6.15a showing the expression of CD69 on purified

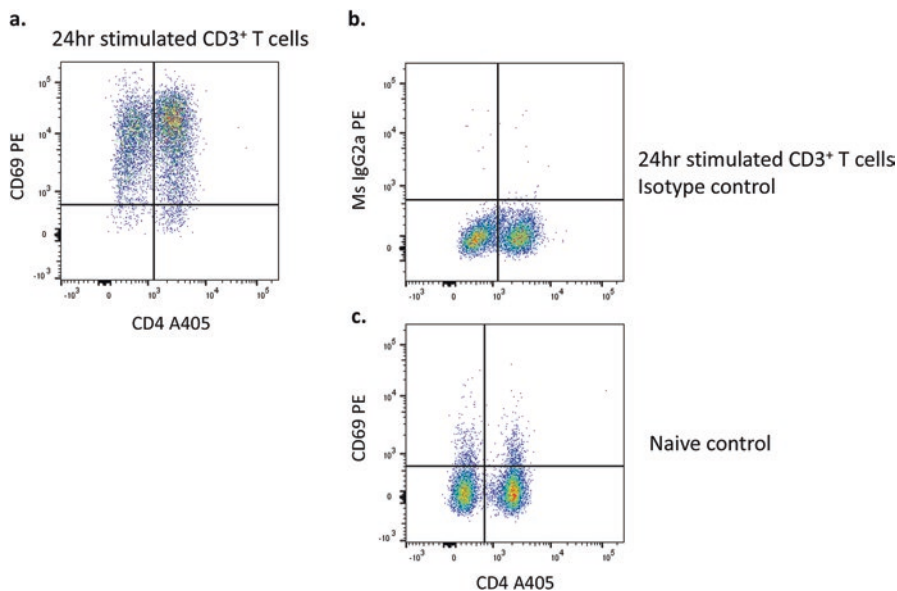


Fig. 6.15 Using isotype gates to determine positive and negative signals on in vitro-activated T cells. Human CD3⁺ T cells were stimulated with Mouse anti-hCD3 (2.5 $\mu\text{g}/\text{mL}$) for 24 h after which the expression of CD69 was determined. In (a) it is impossible to determine the correct position for the negative gate as no distinct negative population exists. Using an isotype control antibody (b) or naive cell control (c) are important for establishing positive and negative gates when examining a uniformly expressed activation marker on a homogenous population of cells

CD4⁺ T cells after being stimulated in vitro with an anti-CD3 antibody for 24 h. We know that CD69 is a T cell activation marker and should be expressed at high levels rapidly after T cells have been stimulated. But how do we know whether the staining pattern we are seeing is truly showing high levels of CD69? How do we know that the voltage for the PE channel wasn't set too high making the level of CD69 on our cells appear artificially too high? This is difficult because in this experiment we don't have an internal negative population like we do when we stain for lineage markers. This can be the challenge of interpreting in vitro experiments because it is assumed that all the cells are being stimulated at the same time and should be expressing the same activation markers at the same time during our experiment. In other words, the cellular responses should be synchronous.

One way to set our negative gate would be to include a sample of naive, unstimulated cells in our experiment since we would assume that naive cells should not express activation markers. For a marker such as CD69, this would work relatively well as can be seen in Fig. 6.15c. When collected at the same PE voltage settings there is a clear difference between the CD69 expression observed on naive cells (CD69^{lo}) compared to what we see at 24 h post stimulation (CD69^{hi}).

Alternatively, we could use an FMO control (discussed below in Sect. 3.4) or we could also compare our naive staining with an appropriate isotype control antibody as shown in Fig. 6.15b. In this case, using either approach will give you very similar results as the negative CD69 gate would be placed approximately half way between the second and third decades along the Y-axis. The MFI of the naive population and the stimulated isotype control stained population is similar and the expression of CD69 on stimulated cells is so high that the percentage of CD69 positive cells will not differ significantly across the two gating strategies. Each strategy has its advantages and disadvantages and these need to be weighed against the intended use for the chosen marker.

Using naive cells is an effective way of determining how the activation status of a cell can affect the expression of a marker; however, as cells become activated, they blast (get bigger) as they divide and begin to express many other proteins which can interact both specifically (i.e., Fc receptors) or nonspecifically with antibodies and fluorochromes, which can, at times, lead to artifactual data. A naive cell control cannot account for all of these changes. Naive cell controls can also be limited to the access to naive cells. This is not as much of a problem when using rodent models as a group of naive animals could be added to each experimental timepoint but it can be a problem when using human samples where obtaining blood or tissue samples multiple times during the course of a study may not be possible. Also, naive cells typically do not survive long when left unstimulated so a naive control many only be possible for 1 or 2 days after the beginning of a study. In experiments where the differences in expression of a receptor are dramatic between naive, unstimulated cells, and activated or treated cells, either naive or isotype controls may be sufficient. When the difference in expression is much more subtle and slight changes in your negative gate can result in large changes in percent positive expression, multiple controls, including non-flow cytometry controls (e.g. PCR), should be used to ensure accuracy of the data.

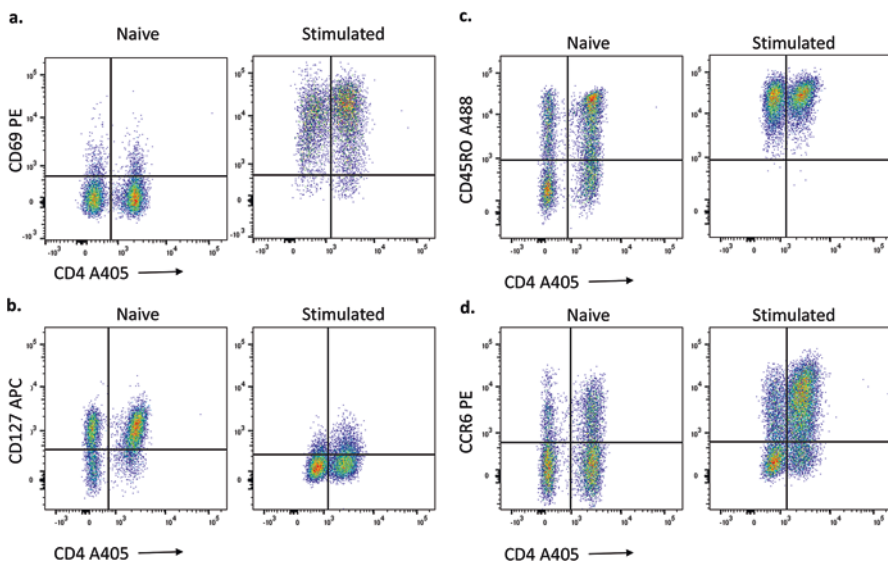


Fig. 6.16 Expression profiles of four different activation markers. Four main types of activation markers can be found: (a) activation markers that are uniformly low on resting cells but get uniformly expressed on activated cells (e.g., CD69), (b) activation markers that are uniformly high on resting cells but get uniformly downregulated on activated cells (e.g., CD127), (c) activation markers that show a bimodal distribution on resting cells but are uniformly up- or downregulated on activated cells (e.g., CD45RO), (d) activation markers that show a bimodal distribution on resting cells and maintain a bimodal distribution during cell activation (e.g., CCR6).

Another challenge with trying to identify the expression of activation markers without the use of naive cells, FMO, or isotype controls is that the expression of activation markers on naive cells is not always low, allowing for the identification of a natural negative population. In the case of CD69 (Fig. 6.16a), it is typical to see uniformly low expression of these markers on naive and resting memory T cells and uniformly high expression of these markers on activated T cells. In most cases it would be possible to establish a negative gate for CD69 on T cells from either mouse or human blood or tissue as the majority of cells should be resting and, as such, CD69^{lo}. Even in the case of blood or tissue samples from mice or humans in the midst of an active immune response, a significant proportion of T cells will still remain unactivated and CD69^{lo}, resulting in a bimodal CD69 distribution, and making the use of an isotype control antibody less important. The use of an isotype control antibody becomes more necessary when T cells are activated in vitro and all cells uniformly express CD69 (Figs. 6.15 and 6.16a). Alternatively, gates set on naive cells can be used keeping in mind the caveats mentioned earlier.

CD127 (Fig. 6.16b) or the alpha chain of the IL-7 receptor can also be considered an activation marker as its expression changes upon T cell activation. What makes this marker different from CD69 is that it is typical to see uniformly high expression of this marker on naive or resting memory T cells and uniformly low expression of

this marker on activated T cells. Using an isotype control or FMO control would be appropriate here. The other challenge with this receptor is that it is not expressed as highly as other activation markers such as CD69 meaning that the difference between a CD127⁺ and CD127⁻ cell is not as dramatic. Following in vitro stimulation, T cells will become uniformly CD127^{lo} so it's important that voltage of the channel used to detect CD127 is sufficiently high to clearly separate CD127^{hi} and CD127^{lo} cells without completely burying the CD127^{lo} cells in the negative decades. An isotype control or FMO control will help prevent this from occurring. In vivo, it's not as likely to observe a clear bimodal distribution in CD127 expression, rather, the transition from CD127^{hi} to CD127^{lo} can appear as more of a continuous smear.

CD45RO is expressed on memory T cells but not on naive T cells. As such, its expression changes as T cells transition from naive to memory cells after T cell activation [3]. In the case of human peripheral blood, we can find both high (memory) and low (naive) expression, or a bimodal distribution of CD45RO on T cells making it relatively straightforward and appropriate to set a negative gate without using an isotype control antibody (Fig. 6.16c). This changes upon in vitro activation as all T cells become CD45RO positive. Using a gating strategy similar to those described for CD69 will work for activated T cell cultures.

Of the four examples in Fig. 6.16, CCR6 represents the only marker for which gating could be done solely by using the bimodal distribution of the receptor on both naive/resting and activated cells (Fig. 6.16d). CCR6, the receptor for macrophage inflammatory protein-3 α (MIP-3 α), is preferentially expressed on memory T cells, and then, preferentially on the CD4⁺ subset [4]. This results in two separate bimodal distributions that can be compared: (1) naive versus memory cells, and (2) CD4⁺ versus CD8⁺ T cells. Both of these distributions can be seen on fresh human PBMCs making it easy to establish a negative gate for both T cell subsets without needing an isotype control antibody. Similarly, the bimodal distribution pattern carries over to activated T cells with CCR6 being preferentially expressed on CD4⁺ T cells. Establishing a negative gate would be more difficult if only CD4⁺ T cells were being analyzed as the population of CCR6^{lo} CD4⁺ T cells is small and the pattern of CCR6 expression is more of a smear than two separate populations. However, because the CCR6^{lo} CD8⁺ T cell population is more well defined, we can use that T cell subset to establish the negative gate for both CD4⁺ and CD8⁺ T cells.

As we've been discussing, when performing in vitro experiments, many times all the cells are being stimulated at the same time and should be expressing the same activation markers at the same time during our experiment. This would necessitate using a combination of naive and isotype controls because there can be a lack of both positive and negative populations depending on the proteins being analyzed. However, during most in vivo experiments, or in vitro experiments using a specific peptide antigen such as a peptide derived from a virus, only a small percentage of cells will likely become activated.

If T cell responses to a specific antigen are being studied, such as transgenic T cells in mice specific for ovalbumin peptide or human T cells specific for influenza peptides, both positive and negative cell populations will exist in your sample. Since the frequency of T cells specific for any given antigen is low, even in artificial adop-

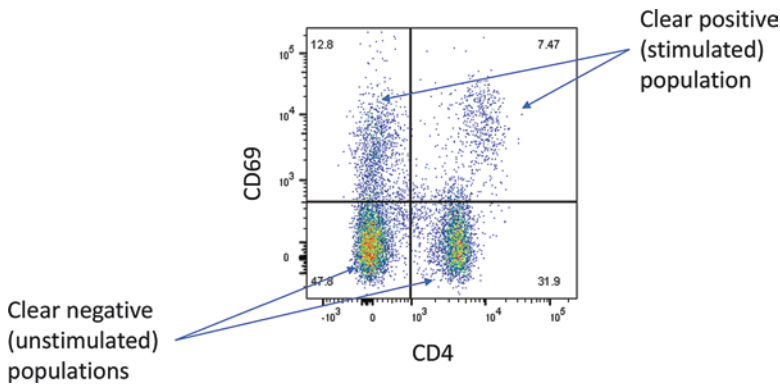


Fig. 6.17 Use of internal naive populations to establish negative gates for activation markers. To artificially create a model where only a small percentage of human T cells become activated, one group of human CD3⁺ T cells was stimulated with Mouse anti-hCD3 (2.5 µg/mL) for 24 h. A second group of CD3⁺ T cells from the same donor was left unstimulated for 24 h. The two groups of T cells were mixed at a 1:4 ratio (1 activated T cell to every 4 unstimulated T cell). The expression of CD69 was then determined on the mixed population.

tive transfer systems, the majority of the T cells will remain inactivated while a small percentage will respond to antigenic stimulation. To illustrate this, Fig. 6.17 shows a group of purified human T cells which have been stimulated for 24 h and then mixed with naive cells from the same donor at a ratio of 4 naive cells to every 1 activated cell. When stained with antibodies to CD69 and CD4 we see that the majority of the cells are CD69^{lo} providing an internal negative population, while a small fraction, the preactivated cells, is CD69^{hi} high. Using this internal negative population, a negative gate could be accurately established without using an isotype control antibody. This strategy may not work with all cell types such as innate immune cell types, which can be activated in a non-antigen-specific manner. Moreover, the distribution of activated/inactivated cells will also vary with location with sights of inflammation or active immune responses being less likely to possess inactivated cells with which to serve as internal negative populations.

3.4 Fluorescence Minus One (FMO)

In some instances, there may not be a good control for the antibodies in your panel. You may not have an isotype control, or you may not have a good internal negative control. It is also not uncommon for some researchers to use Fluorescence Minus One (FMO) exclusively. FMO can be defined as including all the antibody conjugates in your panel minus one of them (no stain). We will go over 3 different examples for FMO. In the first example, mouse splenocytes have been surface stained with a 3-color flow panel; the surface markers include CD4 A405, NK1.1 PE, and CD127 A488. CD4 is a lineage marker so no isotype control is needed. The isotype

Table 6.2 Using FMO for detection of surface markers in mouse splenocytes

Tube	CD4 A405	CD127 A488	NK1.1 PE
Isotype controls	+	Rat IgG2A A488	Mouse IgG2A PE
A488 FMO	+	–	+
PE FMO	+	+	–
Antibody panel	+	+	+

controls, FMO controls, and the corresponding tube with the full panel of antibodies are outlined in the table below (Table 6.2):

Using the isotype control tube, you would set your gate for NK1.1 PE and CD127 A488 based on Mouse 2A PE and Rat IgG2A A488, respectively. In the A488 FMO tube, you would set your gate for CD127 A488 based on no stain; NK1.1 PE and CD4 A405 are present. In the PE FMO tube, you would set your gate for NK1.1 PE based on no stain; CD127 A488 and CD4 A405 are present. The antibody panel tube contains all three antibodies or CD4 A405, CD127 A488, and NK1.1 PE.

Consider the differences in gating using FMO versus isotype controls for NK1.1 PE and CD127 A488 (refer to Fig. 6.18a). The FMO negative control gates are lower due to fluorescence spread that is common with conjugated isotype controls [5]. Using FMO, you inadvertently include some CD4⁺ T cells that should be negative for NK1.1 PE because the quadrant is set higher. The percentage of CD4⁺NK1.1⁺ cells changes from ~3% using the isotype control gate to ~12% using the FMO control gate. CD4⁺CD127⁺ expression using the FMO versus isotype control gate is virtually identical (30.7% vs. 27.9%, respectively). However, CD4[–] T cells have increased expression of CD127 A488 using the FMO gate versus using the isotype gate (~37% versus 13%, respectively). Overall, there is increased background staining using FMO controls over isotype controls.

Table 6.3 outlines the setup using FMO with secreted cytokines in naive versus stimulated human monocytes (Monensin only versus Monensin + LPS, respectively).

In this example, setting gates using FMO for GRO α PE is misleading in naive monocytes. Refer to Fig. 6.18b, and you can see that GRO α PE expression in naive CD14⁺ monocytes is much higher using the FMO negative control (61.3%) over the isotype control (10.0%). This becomes problematic if percentages are the sole basis of comparison for GRO α expression in stimulated (see Fig. 6.18c) versus naive CD14⁺ monocytes; they are identical or 61.3%. Naive IL-6 levels aren’t as dramatically affected using the FMO versus isotype control gates (35.4% vs. 23%, respectively). Stimulated versus naive levels of IL-6 expression using FMO gates are still quite different (59.7% vs. 35.4%); stimulation induces IL-6 expression as expected. However, the fold induction using FMO controls over isotype controls is different; 1.7-fold versus 2.5-fold, respectively. Both examples illustrate that FMO controls aren’t perfect as unstained samples typically stain lower than an isotype control, but it is a good reference point if you have nothing else to use. FMO is an acceptable practice; however, you may have some false positive staining relative to what you would see if you had set your negative gates using isotype controls. Consult the literature so you are familiar with the staining patterns of the antibodies in your panel.

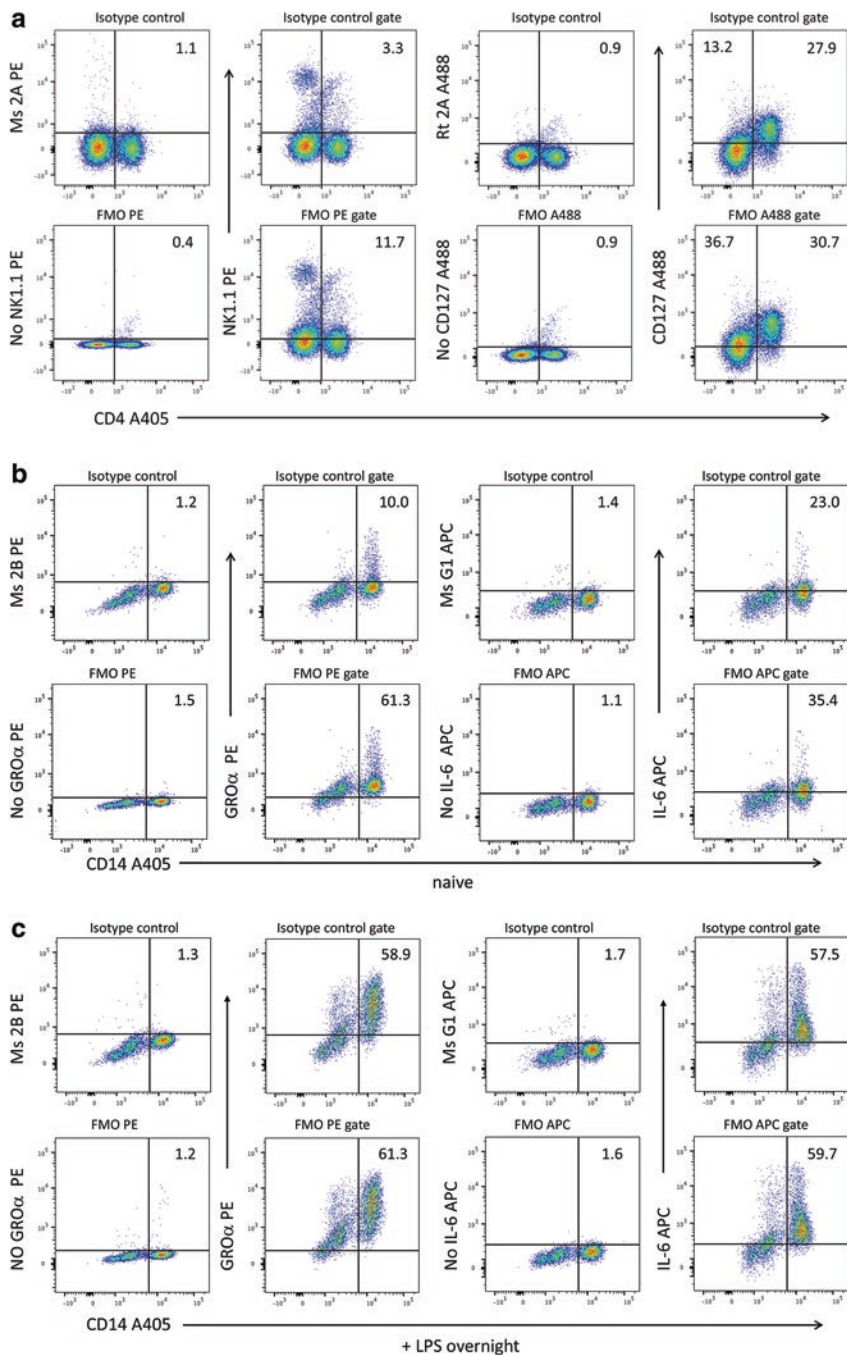


Fig. 6.18 Using Fluorescence Minus One for surface and intracellular markers in mouse and human cells. **(a)** Mouse splenocytes were harvested from C57Bl/6 mice, and stained with mCD4 A405, mCD127 A488, and mNK1.1 PE. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software. **(b, c)** PBMCs were cultured overnight in the presence of Monensin only (3 μ M, **b**) or LPS (1 μ g/mL) + Monensin (3 μ M, **c**). Cells were harvested and stained with hCD14 A405, hGRO- α PE, and hIL-6 APC. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software.

Table 6.3 Using FMO for detection of secreted cytokines in human monocytes

Tube	CD14 A405	IL-6 APC	GRO- α PE
Isotype controls	+	Mouse IgG1 APC	Mouse IgG2B PE
APC FMO	+	–	+
PE FMO	+	+	–
Antibody panel	+	+	+

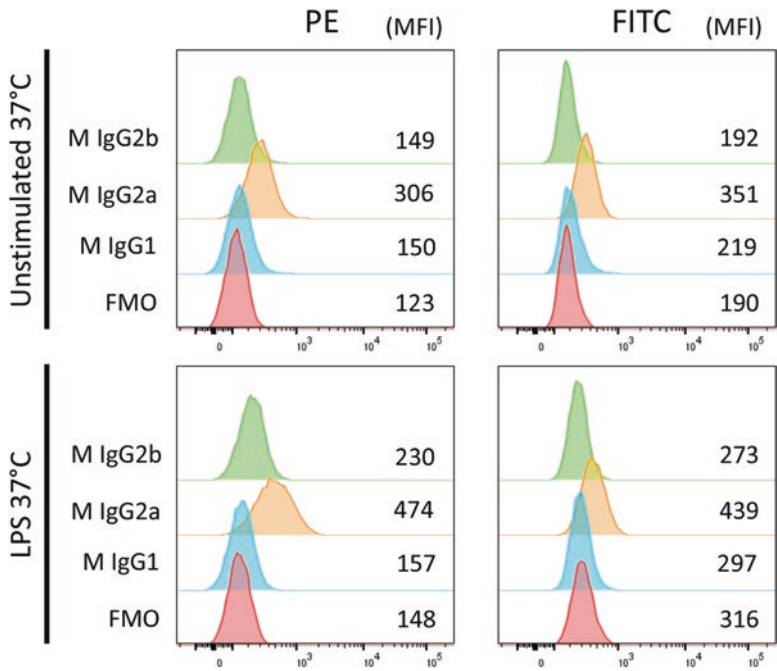


Fig. 6.19 Background surface staining of mouse IgG1, IgG2a, and IgG2b isotype control antibodies compared to FMO controls on human monocytes. Human PBMC was stimulated with 20 ng/mL IFN γ + 20 ng/mL LPS for 12 h at 37 °C or was left unstimulated. Cells were then stained with hCD14, to identify monocytes, and with mouse IgG1, IgG2b, or IgG2a antibodies conjugated with either PE (left column) or FITC (right column). Some cells were only stained with hCD14 to generate FMO controls for PE and FITC. The MFI for PE and FITC for each isotype control antibody was determined and compared to the FMO control for each fluorescence channel. Red histograms represent FMO controls for both PE and FITC. Blue histograms represent Ms IgG1 staining, orange histograms represent Ms IgG2a staining, and green histograms represent Ms IgG2b staining.

When properly titrated, the background fluorescence from the isotype control should be virtually equivalent to an FMO control but this isn't always the reality. In the next section we will discuss Fc receptors and how non-antigen-specific binding of flow antibodies to Fc receptors can lead to increases in background fluorescence. This can be affected by both the isotype of the antibody, the fluorochrome, and the activation state of the cell. As shown in this third example (Fig. 6.19), Mouse IgG1

antibodies yield a low amount of background fluorescence (MFI) that is comparable to their unstained counterpart (FMO) on both unstimulated and LPS stimulated human monocytes whether they are conjugated to FITC or PE. However, notice how the MFI for both the FMO control and Mouse IgG1 isotype control are higher on LPS stimulated monocytes compared to unstimulated monocytes. A similar statement can be made for Mouse IgG2b antibodies although here we see that the background fluorescence of a Mouse IgG2b PE-conjugated antibody is higher than a Mouse IgG2b FITC-conjugated antibody on LPS-stimulated cells. When using a bright fluorochrome such as PE or APC, even a small amount of nonspecific “stickiness” is more noticeable than when using a dimmer fluorochrome such as FITC. Mouse IgG2a antibodies are particularly messy on human monocytes and macrophages given the affinity of the human CD64 Fc receptor for Mouse IgG2a antibodies. The MFI for both the FITC and PE-conjugated Mouse IgG2a isotype antibody is higher than both the Mouse IgG1 and IgG2b antibodies and is more than three times higher than the FMO control on LPS-stimulated monocytes when using the PE-conjugated version.

Using an FMO in this situation would be correct as it would provide you with a cleaner negative population. In this example the cells were not treated to block Fc receptors and the nonspecific stickiness inherent with certain isotypes of antibodies can be seen. Remember, as cells become activated and start dividing they become bigger and may have increased background fluorescence intensity because of their size. The upregulation of other Fc receptors or heavily glycosylated proteins that are naturally “sticky” may also nonspecifically bind antibodies leading to artifactual staining so it’s always a good idea to include an isotype control antibody along with any FMO controls at least until you are familiar with your system and know what pitfalls to expect. Correct cell preparation involving the blocking of Fc receptors can help alleviate these nonspecific stickiness issues.

4 Materials

4.1 Flow Antibodies

- Mouse CD4 A405 (FAB554V; R&D Systems)
- Mouse CD127 A488 (FAB7473G; R&D Systems)
- Mouse NK1.1 PE (FAB8319P; R&D Systems)
- Human CCR6 PE (FAB195P; R&D Systems)
- Human CD3 (MAB100; R&D Systems)
- Human CD4 (MAB3791; R&D Systems)
- Human CD4 A405 (FAB3791V; R&D Systems)
- Human CD14 A405 (FAB3832V; R&D Systems)
- Human CD45RO A488 (BioLegend)
- Human CD69 PE (FAB23591P; R&D Systems)

- Human CD127 APC (FAB306A; R&D Systems)
- Human GRO-alpha PE (IC275P; R&D Systems)

4.2 Isotype Control Antibodies

- Mouse IgG1 FITC (IC002F; R&D Systems)
- Mouse IgG1 PE (IC002P; R&D Systems)
- Mouse IgG2a FITC (IC003F; R&D Systems)
- Mouse IgG2a PE (IC003P; R&D Systems)
- Mouse IgG2b FITC (IC0041P; R&D Systems)
- Mouse IgG2b PE (IC0041P; R&D Systems)
- Mouse IgG1 APC (IC002A; R&D Systems)
- Rat IgG2a A488 (IC006G; R&D Systems)

4.3 Secondary Antibodies

- Goat anti-mouse PE (F0102B; R&D Systems)

4.4 Cell Activation Reagents

- MxhCD3 (MAB100; R&D Systems)
- GtxhCD28 (AF-342-PB; R&D Systems)
- Monensin (Sigma)
- LPS (Sigma)

4.5 Staining Buffers/Fc Block Reagents

- Normal Human IgG (1-001-A; R&D Systems)
- Mouse IgG
- Flow cytometry staining buffer (FC001; R&D Systems)
- 1× Fixation buffer (FC004; R&D Systems)
- 1× Saponin (FC005; R&D Systems)
- Flow Cytometry Human Lyse Buffer (FC002; R&D Systems)

References

1. Uhlen M, Bandrowski A, Carr S, Edwards A, Ellenberg J, Lundberg E, Rimm DL, Rodriguez H, Hiltke T, Snyder M, Yamamoto T. A proposal for validation of antibodies. *Nat Methods*. 2016;13(10):823–7.
2. Baker M. Reproducibility crisis: blame it on the antibodies. *Nature*. 2015;521:274–6.
3. Ohara T, Koyama K, Kusunoki Y, Hayashi T, Tsuyama N, Kubo Y, Kyoizumi S. Memory functions and death proneness in three CD4⁺CD45RO⁺ human T cell subsets. *J Immunol*. 2002;169:39–48.
4. Kleinewietfeld M, Puentes F, Borsellino G, Battistini L, Rotzschke O, Falk K. CCR6 expression defines regulatory effector/memory-like cells within the CD25(+)CD4⁺ T-cell subset. *Blood*. 2005;105(7):2877–86.
5. Roederer M. Compensation in flow cytometry. *Curr Protoc Cytom*. 2002;22(1):1.14–1.14.20.

Chapter 7

Experimental Considerations with Data Sets as Examples



Christopher Hammerbeck, Christine Goetz, Li Jen Peng, and Jae-Bong Huh

1 Fc Receptor Blocking

In humans and rodents, many immune cell types express Fc receptors, which upon binding to antibodies can effect many cell functions such as degranulation, phagocytosis, antibody-dependent cell-mediated cytotoxicity, and the release of cytokines such as IFN- γ . For the purpose of our examples, we are going to focus on Fc receptor expression on monocytes and macrophages (refer to Fig. 7.1).

Fc receptors bind the Fc (constant) portions of antibodies/immunoglobulin (Fig. 7.2) and are classified by the class of antibody that they bind.

A list of common Fc receptors can be found in Table 7.1 [1, 2]. Since most flow cytometry antibodies are isotypes within the IgG class, we are most interested in the Fc-*gamma* receptors.

The list of the most commonly known members of the Fc gamma receptor class are CD64, CD32, and CD16. Fc gamma receptors typically bind IgG immune complexes and do so with relatively low affinity (Fig. 7.3). However, CD64, which is constitutively expressed on monocytes and macrophages, has the ability to bind monomeric IgG as does CD16 when expressed on NK cells. In addition to binding monomeric human IgG, human CD64 also shows a particular affinity for cyanine dyes and mouse IgG2a antibodies.

Blocking Fc receptors on cells is relatively straightforward and is done prior to adding flow cytometry antibodies. Many commercially available Fc Block reagents can be purchased for this purpose. These are generally blocking antibodies for a

C. Hammerbeck · C. Goetz (✉) · L. J. Peng · J.-B. Huh
Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA
e-mail: Chris.Hammerbeck@bio-technie.com; Christine.Goetz@bio-technie.com;
Li.Peng@bio-technie.com; Jae-Bong.Huh@bio-technie.com

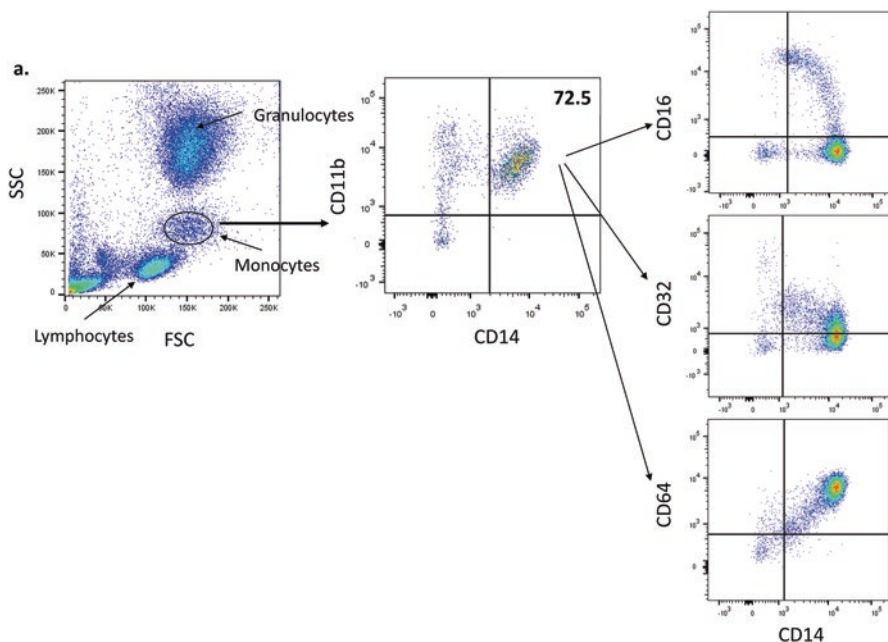


Fig. 7.1 Expression of common Fc receptors on human monocytes. Monocytes were identified in human whole blood (a) by gating on hCD11b and hCD14 double positive cells (b). Monocytes were then analyzed for the expression of the Fc γ receptors hCD16, hCD32, and hCD64 (c)

Fig. 7.2 Basic structure of an antibody. The variable regions of the heavy and light chains are shown in black while the conserved regions are shown in red



given set of Fc receptors. Alternatively, you can use IgG of the same species to block the cells you are staining. In other words, if we are staining human monocytes, you can incubate them with human IgG Fc prior to adding your flow cytometry antibodies. On human monocytes, when Fc receptors are not blocked, they have the potential to bind to the Fc regions of flow cytometry antibodies, especially those that are of the mouse IgG2a isotype and create false positive results (Fig. 7.4a). When an Fc block reagent or IgG are added, they block the binding regions of the Fc receptors, preventing them from interacting with flow cytometry antibodies (Fig. 7.4b).

But does this really matter? While the concept of blocking Fc receptors is simple, the result can mean the difference between detecting or missing a true positive signal or under estimating phenotypic changes in activated cells. In Fig. 7.5 we compare the expression of SLAM F7 on unpolarized M0 and IFN γ and LPS polarized M1 macrophages. The Ms anti-human SLAM F7 antibody is conjugated to APC

Table 7.1 Common human Fc receptors

Fc receptor	CD designation	Affinity	Class	Immunoglobulin specificity	Cellular expression
FcεRI	CD89		Alpha	IgA	Monocytes/neutrophils/Eosinophils
FcεRI			Epsilon	IgE	Eosinophils/basophils/mast cells/Langerhans cells
FcεRII	CD23		Epsilon	IgE	B cell/T cell/NK cell/dendritic cell/eosinophil/macrophage
FcγRIIIa	CD16a	Low affinity	Gamma	IgG3 > IgG1 = IgG4 > IgG2	NK cell/macrophage/γδ T cell/monocytes
FcγRIIIb	CD16b	Low affinity	Gamma	IgG3 > IgG1 = IgG2	Neutrophil/eosinophil
FcγRIIIa	CD32a	Low affinity	Gamma	IgG1 > IgG3 > IgG2 = IgG4	Monocyte/macrophage/neutrophil/Langerhans cells/platelets
FcγRIIIb	CD32b	Low affinity	Gamma	IgG1 = IgG3 = IgG4 > IgG2	Monocyte/macrophage/B cells/placenta
FcγRI	CD64	High affinity	Gamma	IgG1 = IgG3 > IgG4	Monocyte/macrophage/neutrophil/eosinophil

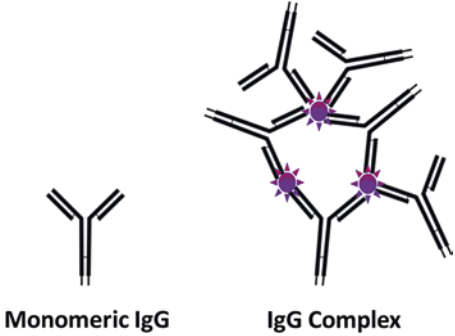


Fig. 7.3 Monomeric IgG versus IgG (immune) complexes. Monomeric IgG are individual antibodies of a given isotype. IgG complexes (aka Immune Complexes—IC) are a group of individual antibodies that are bound to the same antigen (represented in purple). CD64 is the human Fc receptor with high affinity for monomeric IgG while the remaining human Fc receptors typically bind IgG complexes with a low affinity

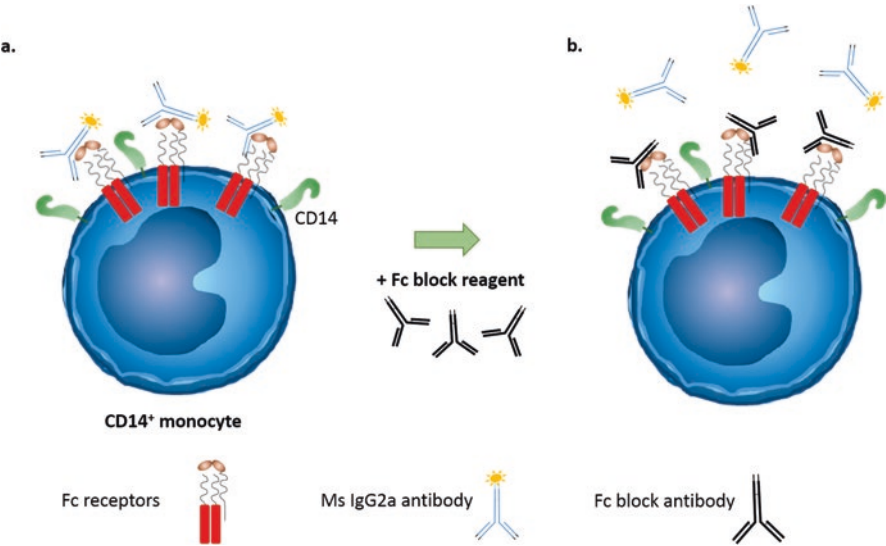


Fig. 7.4 Fc receptor blocking. When Fc receptors are left unblocked, they can nonspecifically bind to flow cytometry antibodies, leading to false positive staining (a). When cells are treated with an Fc block reagent, the Fc receptors on the cell preferentially bind the Fc block reagent, leaving flow cytometry antibodies to bind in an antigen-specific manner (b)

(blue histograms) meaning we will use a Ms IgG2a APC-conjugated isotype control antibody (gray histograms).

First let's compare isotype control staining. When the macrophages are treated with Fc block, there is very little background fluorescence when using the Ms IgG2a isotype control on both M1 and M0 macrophages (Fig. 7.5a, c—gray histograms).

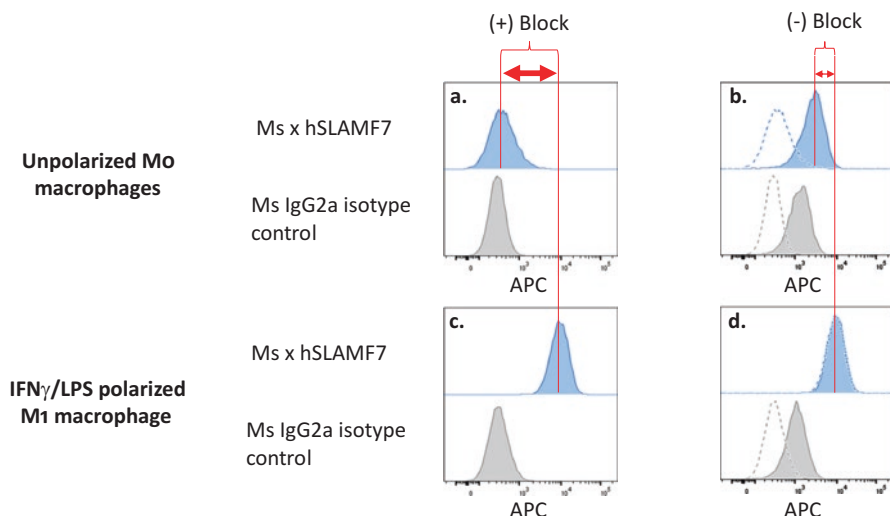


Fig. 7.5 Nonspecific binding of flow cytometry antibodies can hinder phenotypic analysis of cells. Human PBMCs were isolated from whole blood and CD14⁺ monocytes were isolated using a CD14⁺ monocyte MagCelect kit. Monocytes were cultured for 6 days in a 6-well plate with 50 ng/mL rhGM-CSF. Unpolarized M0 (**a** and **b**) and IFN γ /LPS polarized M1 (**c** and **d**) macrophages were stained with a Mouse anti-human SLAM F7 APC antibody (blue histograms) or appropriate Mouse IgG2a isotype control antibody (gray histograms). Both sets of macrophages were either left untreated (**b** and **d**) or were treated with an Fc block reagent for 15 min at 4 °C (**a** and **c**). The fluorescence for the SLAM F7 APC antibody or Ms IgG2a APC antibody is shown. In (**b**) and (**d**), the blue and gray dashed histograms represent the APC fluorescence of unpolarized M0 (**b**) and IFN γ /LPS polarized M1 (**c**) macrophages stained with either the SLAM F7 antibody or Ms IgG2a antibody. The red lines represent the difference in expression between SLAM F7 on unpolarized and polarized macrophages. The difference in expression is significantly reduced when macrophages are not treated with Fc block before staining

Now compare those results to the same macrophages that were not treated with Fc block prior to antibody staining in Fig. 7.5b, d (gray histograms). The dashed gray histograms in Fig. 7.5b, d represent the background fluorescence of the Ms IgG2a antibody on macrophages treated with Fc block. In the absence of Fc block we have a noticeable increase in background fluorescence in the APC channel on both sets of macrophages when using the Ms IgG2a isotype control antibody, represented by the red arrows pointing toward the right.

Let's compare the expression of SLAM F7 on these macrophages. We can see that when treated with Fc block, the expression of SLAM F7 detected on unpolarized M0 macrophages is just barely above that of the isotype control (Fig. 7.5a—blue histogram). In contrast we can see that polarized M1 macrophages express this marker quite highly (Fig. 7.5c—blue histogram). However, in the absence of Fc block, the differences in SLAM F7 expression between M0 and M1 macrophages become less clear, Fig. 7.5b, d (blue histograms). The dashed blue histograms in Fig. 7.5b, d represent the background fluorescence of the SLAM F7 antibody on macrophages treated with Fc block. Notice how, in the absence of Fc block, there is

an increase in the background fluorescence in the APC channel on the unpolarized M0 macrophages when using the SLAM F7 antibody. This is because the SLAM F7 antibody shares the Ms IgG2a background and binds in a non-antigen-specific manner in the absence of Fc block.

The fluorescence of the SLAM F7 APC antibody on polarized M1 macrophages is still higher but the difference in expression of SLAM F7 between the two macrophage subsets is now much more subtle. The red lines laid over each set of histograms represent the difference in the MFI of SLAM F7 expression between M0 and M1 polarized macrophages. Notice how this difference is much smaller in the absence of Fc block.

2 Dead Cell Exclusion

Dead cells can present a real problem for interpreting flow cytometry results primarily because dead cells can take up or nonspecifically bind to flow cytometry antibodies or otherwise autofluoresce which can result in false positive data. So how do we detect dead cells and distinguish them from the live cells we are interested in? One of the most common methods used today is the use of cell viability dyes. Most of these viability dyes can be divided into two categories: dyes that label proteins such as the fixable viability stains and dyes that label nucleic acids such as 7-AAD and Propidium iodide (see Fig. 7.6). Both of these types of dyes are cell membrane impermeable dyes meaning that they will be excluded from live cells, which have intact cell membranes but will be passively taken up by apoptotic cells which have

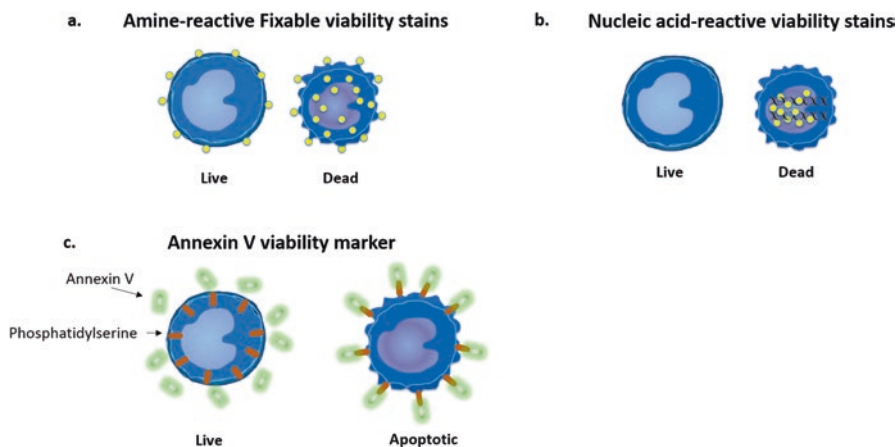


Fig. 7.6 Mechanisms of action of various viability dyes and proteins. Amine-reactive fixable viability dyes rely on cell membrane permeability to stain proteins on the inside of dead cells (a). Nucleic acid-reactive viability dyes rely on cell membrane permeability to stain DNA on the inside of dead cells (b). Annexin V binds to phosphatidylserine that is flipped to the outside of the cell membrane early on in the apoptosis process (c)

compromised membrane integrity. The fixable viability dyes (Fig. 7.6a) are amine reactive dyes that covalently label free amines on intracellular proteins. On live cells, the dyes react with surface proteins, but these are much less abundant than intracellular proteins, resulting in low staining levels compared to dead cells. Nucleic acid dyes (Fig. 7.6b) such as 7-AAD bind to DNA with high affinity for the GC-rich regions. Because these dyes are also nonpermeable to intact cell membranes, they selectively label apoptotic cells. Fixable viability dyes are sufficiently stable such that cells can be labeled before fixation and permeabilization steps to detect intracellular proteins without losing the ability to discriminate between live and dead cells. Nucleic acid dyes such as 7-AAD must be used on unfixed cells shortly before acquiring the samples on the cytometer. Perhaps the biggest drawback of 7-AAD is that it has a very broad emission spectrum with a peak emission wavelength similar to PerCP. Consequently, the fluorescence spillover of 7-AAD into other channels such as APC, FITC, and PE can be quite substantial, and compensation can be tricky.

Another method that can be used is the use of fluorescently labeled Annexin-V protein (Fig. 7.6c). Annexin V is a cellular protein that binds to phosphatidylserine, a phospholipid that is a component of a cell's plasma membrane. On live cells, phosphatidylserine is found on the luminal side of the plasma membrane, however during apoptosis, phosphatidylserine is flipped to the outside of the plasma membrane. Thus, when used as a viability marker, Annexin V will preferentially bind to apoptotic cells while excluding live cells. The flipping of phosphatidylserine to the outside of the plasma membrane precedes the disruption of the plasma membrane which is necessary for the function of the amine- and nucleic acid-reactive viability dyes making it a good marker of early apoptosis.

All three methods of detecting apoptosis can be used simultaneously and detect a similar population of cells, as shown in Fig. 7.7a. Here, KG.1 cells were treated with camptothecin for 12 h to induce apoptosis after which they were labeled with Annexin V (Fig. 7.7b), an amine-reactive apoptosis dye (Fig. 7.7c) and the nucleic acid-reactive apoptosis dye 7-AAD (Fig. 7.7d). Dead cells appear as the cells with the highest fluorescence values for each detection method. Live cells show low fluorescence values for each detection method. When we compare the forward and side scatter profile of dead cells identified by each method, we see that all three methods identified the same FSC^{lo} population of cells closest to the Y-axis (represented in red in Fig. 7.7e–g). Annexin V further labeled a small fraction of cells within the second FSC^{mid-hi} live cell population that were not identified by the amine-reactive apoptosis dye and 7-AAD. These are early apoptotic cells whose membranes are not yet permeable to the other dyes. Thus, one advantage of Annexin V is that it will recognize both early and late apoptotic and dead cells. One consideration when using amine- or nucleic acid-reactive dyes is that variability in the extent of membrane permeability during late apoptosis can, in some cases, lead to variability in the labeling of cells by these dyes.

When used together in Fig. 7.8, Annexin V and the amine-reactive viability dye (Fig. 7.8a) or nucleic acid-reactive viability dye (Fig. 7.8b) are often used to discriminate early apoptotic and late apoptotic/necrotic cells as seen in showing the

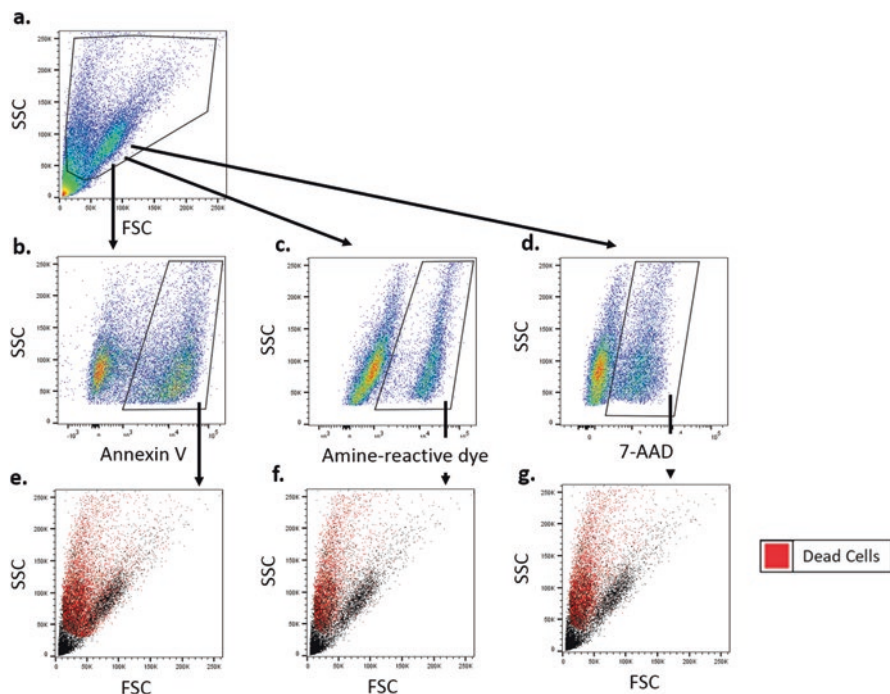


Fig. 7.7 Detection of apoptotic and dead cells by various viability dyes and proteins. KG.1 cells were treated with 5 μ M camptothecin for 12 h to induce apoptosis (a). Cells were then labeled with Annexin V (b and e), an amine-reactive dye (c and f) or 7-AAD (d and g). Dead cells were identified as those cells showing positive fluorescence for Annexin V (b), an amine-reactive dye (c), or 7-AAD (d). The forward and side scatter profiles of the dead cells were determined for Annexin V (e), an amine-reactive dye (f) or 7-AAD (g). All three methods to detect apoptosis labeled the same forward scatter^{lo} population of cells

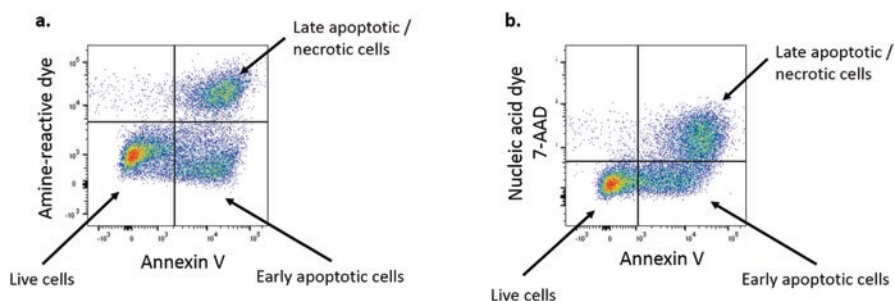


Fig. 7.8 Detection of early and late apoptotic events. Combining Annexin V with either an amine-reactive dye (a) or 7-AAD (b) allows for the discrimination of early and late apoptotic events. Live cells are Annexin V⁻ / amine-reactive dye⁻ and 7-AAD⁻. Early apoptotic cells are Annexin V⁺ and amine-reactive dye⁻ and/or 7-AAD⁻. Dead/necrotic cells are Annexin V⁺ and amine-reactive dye⁺ and/or 7-AAD⁺

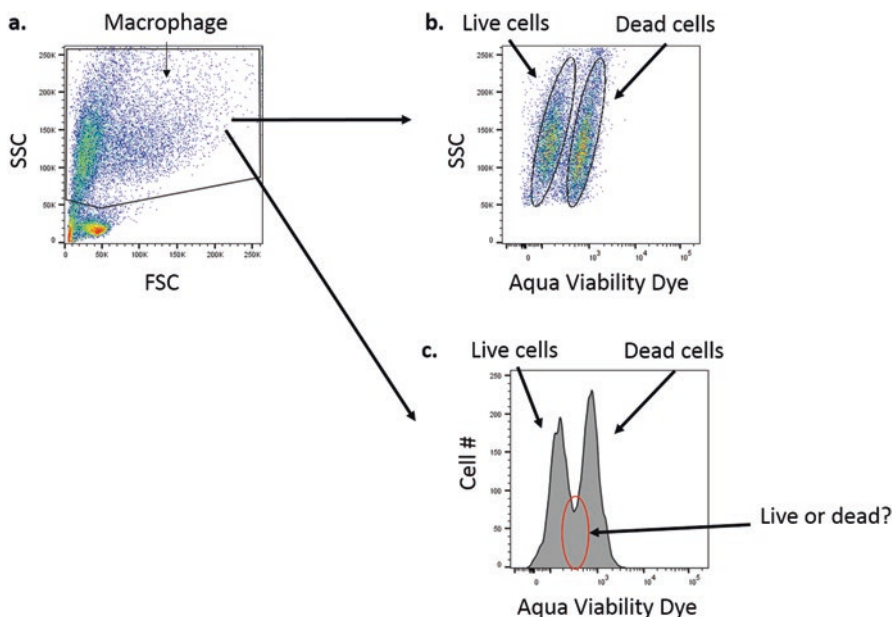


Fig. 7.9 Discrimination of live and dead cells using dot plots or histograms. Human PBMCs were isolated from whole blood and CD14⁺ monocytes were isolated using a CD14⁺ monocyte MagCelect kit. Monocytes were cultured for 6 days in a 6-well plate with 50 ng/mL rhGM-CSF. Human macrophages were polarized with 20 ng/mL IFN γ + 20 ng/mL LPS for 12 h before harvesting and labeling cells with an amine-reactive aqua viability dye (a). Macrophages were analyzed for aqua viability dye fluorescence using (b) a side scatter versus aqua viability dye dot plot or (c) an aqua viability dye histogram. Side scatter versus viability dye dot plots provide better resolution when using dim viability dyes

detection of three cell populations following the induction of apoptosis in KG.1 cells with camptothecin. All three viability markers can be used along with other antibodies to other cellular markers.

Despite the different mechanisms used by the various viability markers to detect apoptosis, the gating strategies to separate live and dead cells is the same for all three. In Fig. 7.9 we are showing IFN γ and LPS polarized human macrophages that have been stained with an amine-reactive fixable aqua viability dye. After gating on the macrophage population in Fig. 7.9a, we can identify the aqua positive dead cells using one of two different plots. The top dot plot (Fig. 7.9b) shows side light scatter (SSC) on the Y-axis and the viability dye on the X-axis. Notice that the SSC is displayed on a linear scale while the fluorescence of the viability dye is represented on a log scale. There are no spectral overlap concerns with the aqua detector on the cytometer when using Side light scatter as a parameter so it is an easy way to separate live and dead cells. We can see that we have two populations of cells: a population on the left which is negative for the viability dye (live cells) and a population on the right which is positive for the viability dye (dead cells).

The bottom plot (Fig. 7.9c) is a histogram showing the representative fluorescence of the aqua viability dye. Here we can also see that we have two peaks or

populations: the peak on the left which is negative for the viability dye (live cells) and the peak on the right which is positive for our viability dye (dead cells). This histogram demonstrates one of the weaknesses of using a histogram with a dim colored dye such as aqua (an AmCyan equivalent) which is excited on the Violet 405 nm laser—there isn't clear separation between the positive and negative peaks. We are left to wonder where do the live cells end and the dead cells begin? In this case, using the top side scatter versus aqua viability dye dot plot gives a better discrimination of the live and dead populations. Viability dyes in other colors such as Alexa 488 or Alexa 647 equivalents are much brighter and will result in two clear distinct peaks when shown as a histogram.

Cell size can often be a good way to start visually distinguishing between live and dead cells as dead cells typically are smaller in terms of forward and side scatter compared to live cells. In Fig. 7.10 CD3⁺ T cells have been stimulated for 24 h with anti-CD3 and CD28 antibodies and two populations are identified within the lymphocyte gate (Fig. 7.10a), a large population on the right side of the gate and a second small population on the left side of the lymphocyte gate which is likely dead based on its small forward and side scatter profile. If these populations are stained with a violet viability dye (Fig. 7.10b) we see that we have a population on the left which is viability dye negative (live cells) and a population on the right which is viability dye positive (dead cells). To determine which of these populations is represented by the small population on the left side of the lymphocyte gate, we would compare the forward and side light scatter profiles of the viability dye negative live and viability dye positive dead cells. If we then overlay the live cells and dead cells back on the original forward and side scatter plot in Fig. 7.10a (a process called back-gating), we can see that the small population on the left side of the lymphocyte gate (represented in red) is in fact the dead cells while the large population on the right side of the lymphocyte gate (represented in blue) is the live cells in Fig. 7.10c.

Size doesn't always matter and sometimes cells that might "appear" dead based on forward or side light scatter may not actually be dead. When live and dead cells occupy the same space, size alone is not sufficient to identify dead cells and a viability dye must be used. Consider the example in Fig. 7.11 showing a population of IFN γ and LPS polarized macrophages. IFN γ and LPS is a potent macrophage stimulus so a high percentage of dead cells could be expected after 24 h. Within the macrophage gate in Fig. 7.11a we can see that virtually all the cells have a low forward scatter and moderate side scatter and look dead. Based on the uptake of an aqua viability dye in Fig. 7.11b we can see that approximately 65% of the macrophages are alive. If we again back-gate the live and dead macrophage populations by overlaying each on the original forward and side scatter plot in Fig. 7.11a, we can see that both the live and dead cells occupy the same forward and side scatter profile and correlate with the bulk of our macrophage population in the top left dot plot Fig. 7.11c. Without the use of a viability dye, we would not have been able to unmask the live cells within this population.

As we mentioned earlier, dead cells can take up or nonspecifically bind to flow cytometry antibodies or otherwise autofluoresce which can result in problematic false positive data. To illustrate this concept let's look again at the polarized human

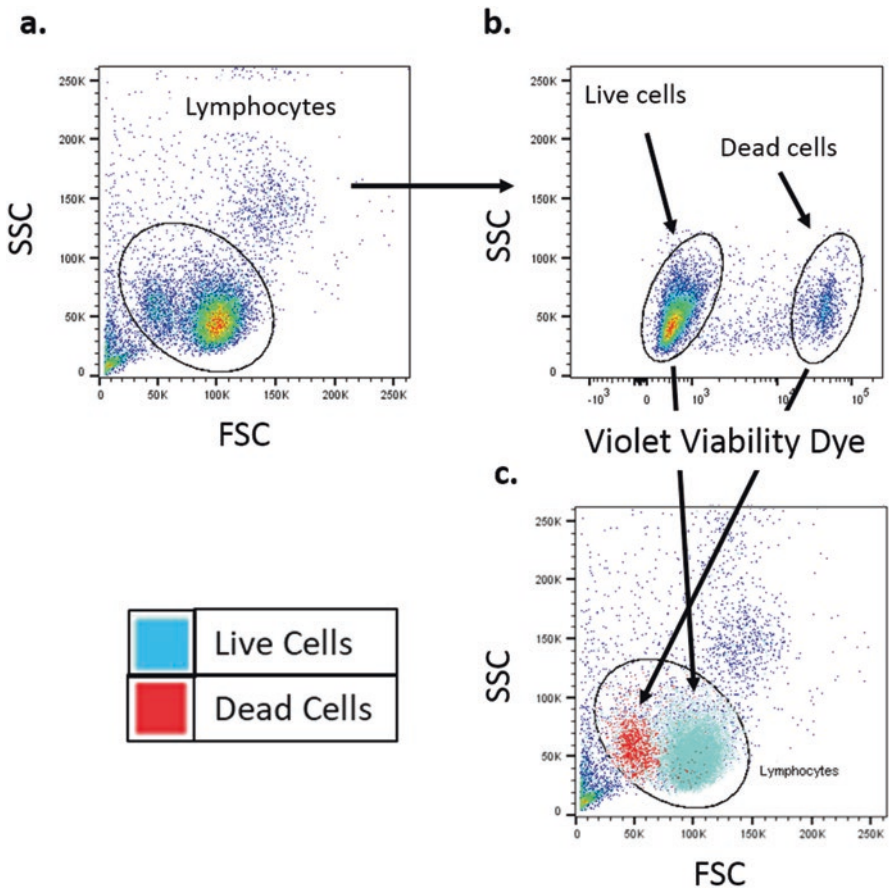


Fig. 7.10 Using size to identify dead cells. Purified human T cells were examined by forward and side scatter. Dead cells were presumed to be a small forward and side scatter^{lo} population on the left of the lymphocyte gate (**a**). To confirm this, cells were labeled with an amine-reactive violet viability dye. Dead cells were identified using a side scatter versus violet viability dye dot plot by gating on the violet fluorescence positive cells (**b**). (**c**) The forward and side scatter profiles for both the violet fluorescence^{lo} (live—blue) and the violet fluorescence^{hi} (dead—red) cells were determined

macrophages from Fig. 7.9 that were labeled with the aqua viability dye. After identifying the live and dead cells in Fig. 7.12a by examining side light scatter and aqua viability dye uptake, we can see that the dead cells in Fig. 7.12b (red histogram) show a higher amount of nonspecific staining with a Mouse IgG2b A700 antibody than do the live cells (blue histogram). Since we are using a non-antigen-specific isotype control antibody which should not bind to the cell, this staining would be considered “false positive” staining. By comparison, both live and dead cells show comparable staining with the Mouse IgG1 A488 antibody (Fig. 7.12c) suggesting that the nonspecific binding of antibodies to dead cells may depend on both antibody isotype and the fluorescent dye used during antibody conjugation.

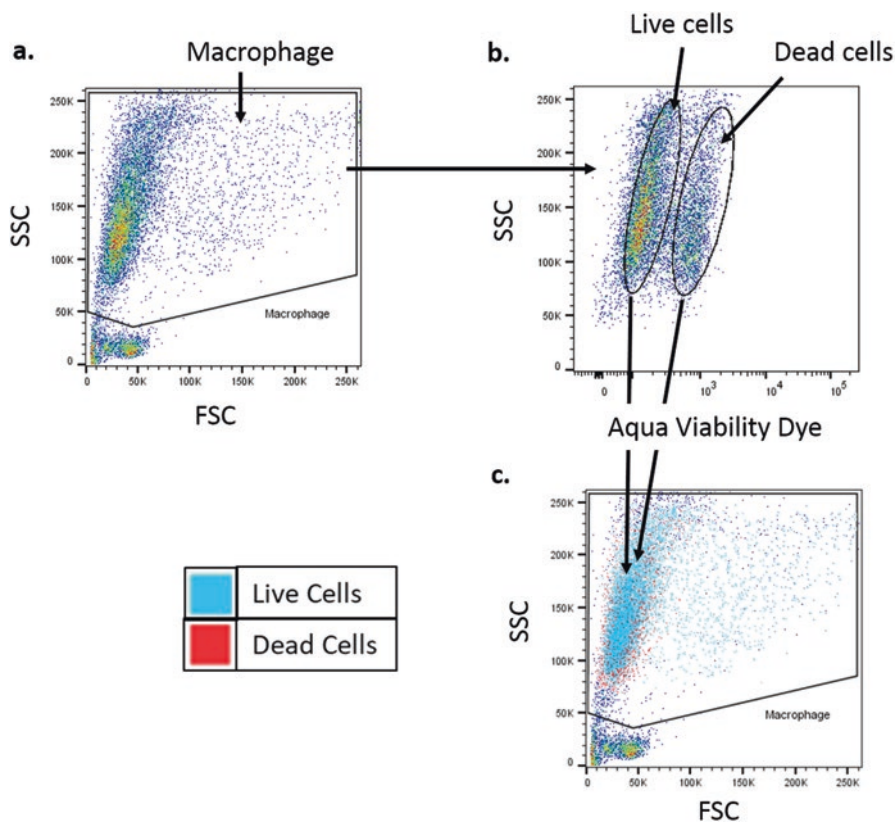


Fig. 7.11 Using viability dyes to distinguish live and dead cells that share a similar size. Human PBMCs were isolated from whole blood and CD14⁺ monocytes were isolated using a CD14⁺ monocyte MagSelect kit. Monocytes were cultured for 6 days in a 6-well plate with 50 ng/mL rhGM-CSF. Human macrophages were polarized with 20 ng/mL IFN γ + 20 ng/mL LPS for 12 h, harvested, and were examined by forward and side scatter. All cells shared a similar forward and side scatter profile (a). Cells were labeled with an amine-reactive aqua viability dye. Dead cells were identified using a side scatter versus aqua viability dye dot plot by gating on the aqua fluorescence positive cells (b). (c) The forward and side scatter profiles for both the aqua fluorescence^{lo} (live—blue) and the aqua fluorescence^{hi} (dead—red) cells were determined

3 Cell Number, Antibody Concentration, and Titration of Flow Antibodies

3.1 Cell Number

During the process of antibody development, each antibody that becomes a product is rigorously tested for specificity and the ideal concentration to use for detecting optimal expression. With that in mind, flow antibodies are typically recommended to be used on 1 million cells/sample. However, counting cells is tedious, especially

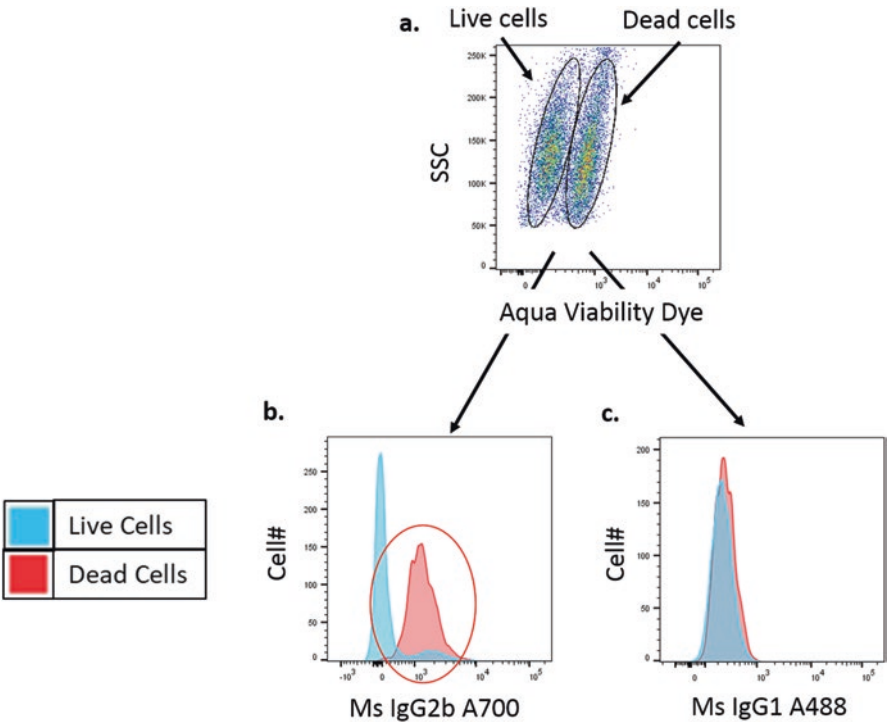


Fig. 7.12 Dead cells nonspecifically bind flow cytometry antibodies. Human PBMCs were isolated from whole blood and CD14⁺ monocytes were isolated using a CD14⁺ monocyte MagCelect kit. Monocytes were cultured for 6 days in a 6-well plate with 50 ng/mL rhGM-CSF. Human macrophages were polarized with 20 ng/mL IFN γ + 20 ng/mL LPS for 12 h before harvesting and labeled with an amine-reactive aqua viability dye (a). Macrophages were then stained with an A700-conjugated Ms IgG2b isotype control antibody (b) or an A488-conjugated Ms IgG1 isotype control antibody (c). Dead cells (red) preferentially bound the A700-conjugated Ms IgG2b isotype control antibody compared to live cells (blue) while failing to nonspecifically bind the Ms IgG1 antibody

when you’re running differentiated cells and have a long reactivation. So, there are instances in which too many cells are stained with your antibody/antibodies of interest. The resulting problem is that you are probably staining too many cells with too little antibody. Refer to the schematic in Fig. 7.13a. With 1 million cells, there is enough antibody relative to the number of cells so all of them are stained. However, with tenfold more cells, many of your cells are not stained, as the antibody wasn’t optimized to stain that many cells so too little antibody is being used.

This is best exemplified in Fig. 7.13b. Varying numbers of CD4⁺ T cells have been stained with CD127 APC at a fixed concentration. CD127 expression is optimal using 1 million cells as shown in the orange histogram. Staining 5 million cells using a fixed amount of CD127 APC decreases the level of CD127 expression substantially. This is because the CD127 APC antibody has been diluted out by using too many cells. This is especially important to keep in mind with low-density markers such as CD127 as expression levels will already be low.

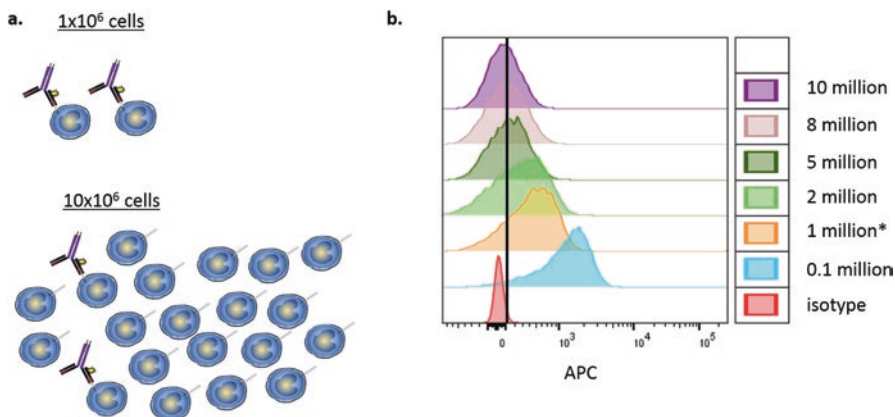


Fig. 7.13 Importance of using the correct PBMC concentration with a fixed amount of CD127 antibody. (a) Schematic showing how using the correct number of cells (top panel) results in optimal antibody staining whereas using too many cells results in poor antibody staining (bottom panel). (b) Human CD4⁺ PBMCs were stained at varying cell concentrations (0.1×10^6 – 10×10^6) using a fixed amount of hCD127 APC. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software

3.2 Antibody Concentration

Another important consideration is the concentration of the antibody you are using. Some antibody companies bottle their products at concentrations higher than what would be required for a flow cytometry experiment and leave it up to the end user to determine the concentration necessary for their model. Other companies bottle their products at a specific concentration and then recommend that a given volume (usually 5–10 µL) is used per test (usually between 5×10^5 – 10^6 cells). What is important to remember here is that in this situation, the bottling concentration is the concentration at which the antibody performed best for the manufacturer using the manufacturer's specific model. That concentration may be suboptimal for the model system that you are using, requiring more or less of a given antibody than recommended. The only way to determine the concentration that you need for a set of experiments is to titrate the antibody. This is often done in a checkboard fashion where varying antibody concentrations are testing against varying cell numbers. Before beginning this type of experiment, it is helpful to determine what you are trying to determine using a given antibody. Are you looking for a simple population distribution of a certain marker or are you trying to determine the extent to which a protein is expressed? These can be two very different questions which can require very different amounts of antibody. Consider the CD62L histograms in Fig. 7.14a. Human PBMCs were stained with varying concentrations of a CD62L antibody, between 1 µg/mL and 200 µg/mL, and the percent of CD3⁺ T cells expressing CD62L was determined. The percent of CD3⁺ T cells expressing CD62L was found to be between 68% and 71% regardless of the concentration of CD62L antibody used. If this was the goal of our experiment, using the CD62L antibody at 1 µg/mL would

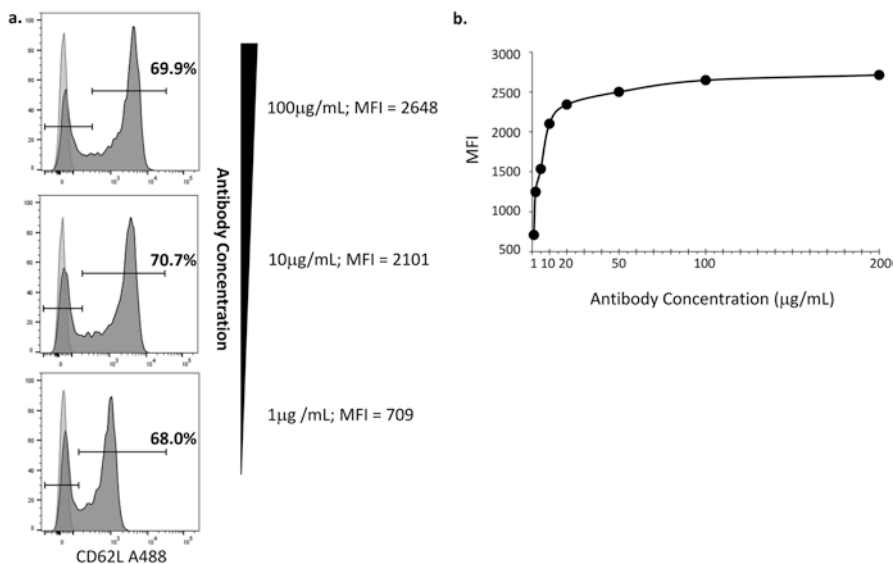


Fig. 7.14 Effects of antibody concentration on cell population discrimination versus antigen density/expression analysis. Human PBMCs were stained with antibodies to hCD3 and hCD4 along with titrated amounts of a hCD62L A488 antibody (1 µg/mL–200 µg/mL). The percentage of CD3⁺ CD4⁺ T cells expressing hCD62L was equivalent across all concentrations of hCD62L A488 tested (a). The MFI of hCD62L was dose dependent (b). While 1 µg/mL hCD62L A488 was sufficient to separate CD62L⁺ and CD62L⁻ cells, much higher concentrations of hCD62L A488 were needed to maximally detect CD62L expression

be sufficient; higher concentrations would be wasting antibody. However, it's plainly obvious that the MFI of CD62L expression increases as the antibody concentration increases. Consequently, if we are interested in the extent that CD62L is expressed on CD3⁺ T cells, we would have to use a higher concentration, or a saturating concentration, of antibody. If we plot the changes in MFI versus the antibody concentration in Fig. 7.14b, we can see that the curve starts to plateau around 20 µg/mL but stabilizes closer to 200 µg/mL; at least 200-fold higher than the concentration necessary to determine population statistics. Without a saturating concentration of antibody, it would be difficult to determine if a variation in protein expression was due to a particular treatment or activation status or simply due to an insufficient amount of antibody being used.

3.3 *Titration of Flow Cytometry Antibodies*

One danger associated with using antibodies at increasingly higher concentrations is that you risk a greater occurrence of nonspecific binding resulting in increased background staining. This isn't obvious using the CD62L antibody in Fig. 7.14 but is nicely illustrated in Fig. 7.15 using a CD25 antibody. CD25 is not typically

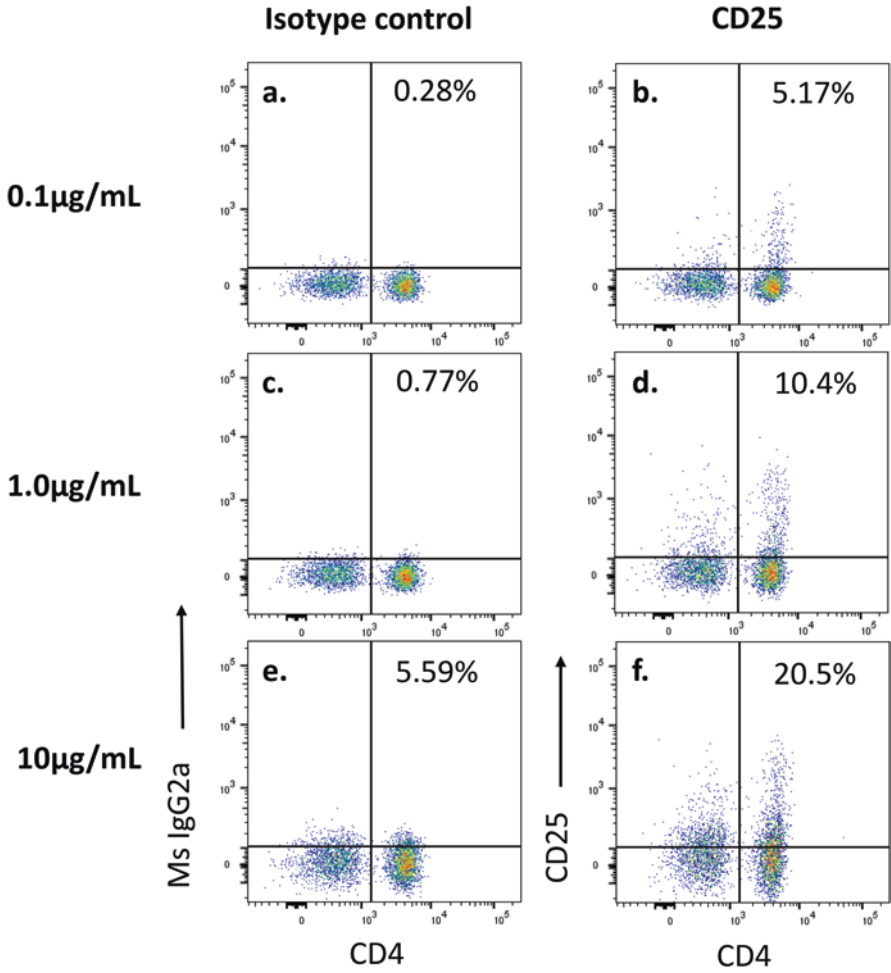


Fig. 7.15 Effects of antibody concentration on background fluorescence of negative populations. Human PBMCs were stained with antibodies against hCD3 and hCD4 along with titrated amount of a hCD25 APC antibody or corresponding Ms IgG2a isotype control antibody (0.1 $\mu\text{g/mL}$ –10 $\mu\text{g/mL}$). The CD25 negative population becomes elongated when using progressively higher concentrations of either Ms IgG2a APC (a, c, e) or hCD25 APC (b, d, f). Background fluorescence of the CD25 negative population is minimal when hCD25 APC is used at 0.1 $\mu\text{g/mL}$ (b) but becomes higher at 1 $\mu\text{g/mL}$ (d) and 10 $\mu\text{g/mL}$ (f) leading to errant increases in the percentage of CD25⁺ cells in resting human CD4⁺ T cells

expressed on resting T cells, as such, T cells from healthy individuals should be largely devoid of CD25 staining. The exception to this is natural regulatory T cells (Tregs) which express intermediate levels of CD25 and which represent 5–7% of the T cell population. Therefore, as we look at Fig. 7.15, we would expect that when stained with a CD25 antibody, the CD4⁺CD25⁺ population in the upper right

quadrant of the dot plots should be 5–7% of all the cells in that plot. When correctly titrated, the amount of background staining displayed by an isotype control antibody should be approximately that of an FMO control.

When used at 0.1 $\mu\text{g/mL}$ we have negligible background staining from the appropriate Mouse IgG2a isotype control antibody (Fig. 7.15a) that is comparable to what we would see using an FMO control. Based on the negative gate, we find that a little over 5% of the T cells are expressing CD25 (Fig. 7.15b; upper right quadrant) and that CD25 expression is restricted to the CD4^+ cells. Not only does this agree nicely with published Treg literature but our negative gate that we established using the Mouse IgG2a antibody matches up nicely with the bimodal CD25 profile that can be observed for CD4^+ T cells; the bulk of the CD4^+ population sits just under the negative gate while the small but distinct $\text{CD25}^+\text{CD4}^+$ population is just above the gate. When used at tenfold higher concentration, 1.0 $\mu\text{g/mL}$, the isotype control plot still looks reasonable (Fig. 7.15c) but now we see that around 10% of the T cells are expressing CD25 (Fig. 7.15d; upper right quadrant). Moreover, as the total MFI for CD25 expression increases, so does the CD25 negative population; instead of being a compact population as seen in Fig. 7.15b, the negative population in Fig. 7.15d is starting to become elongated and now starts to cross over the negative gate. This pattern is further exemplified when using the antibodies at 10 $\mu\text{g/mL}$. Now we can see substantial background staining from the isotype control antibody (Fig. 7.15e) and nearly 20% of the T cells are positive for CD25 (Fig. 7.15f). Both the CD4^+ and CD4^- CD25 negative populations have become elongated and cross over the negative gate making it appear that even a substantial percentage of resting CD4^- (CD8^+) T cells express CD25. A bimodal CD25 expression pattern still exists within the CD4^+ T cells suggesting that we could simply reposition the negative gate higher. While this may work and provide us with the correct percent of CD25 expressing T cells, this would be incorrect in practice as the high background fluorescence seen when using the antibody at 10 $\mu\text{g/mL}$ would far exceed the FMO control and could negatively impact compensation with other fluorochromes.

In summary, blocking your cells for Fc receptors, and excluding dead cells will aid in producing clean and reproducible data. In addition, titrating the amount of cells and antibodies used in your experiments will also help ensure detection of the desired proteins on your cells. These considerations apply to both surface and intracellular staining and are clearly outlined in flow staining protocols which encompass Chap. 9.

4 Surface Staining Considerations

4.1 From 2 to 12 Parameter Flow Cytometry

Many researchers start out by doing 2-parameter flow cytometry to avoid compensation or simply because their experiments do not require multiple markers. However, once you master compensation (Chap. 5), and learn how to choose the optimal fluorochromes for a multiparameter experiment (Chap. 4), it is not as

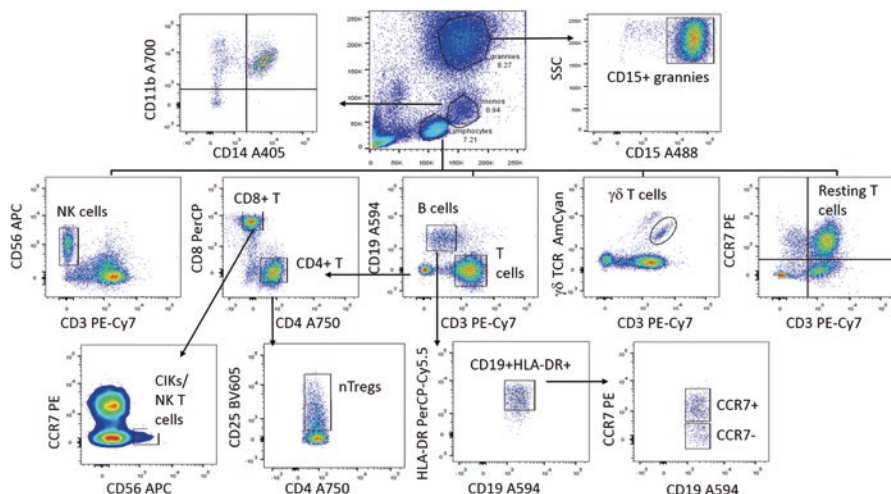


Fig. 7.16 The family tree of human whole blood cells: 12 parameter flow cytometry. Human whole blood was stained with the following panel of surface markers: hCD11b A700, hCD14 A405, hCD15 A488, hCD3 PE-Cy7, hCD4 A750, hCD8 PerCP, hCD19 A594, hHLA-DR PerCP-Cy5.5, hCCR7 PE, h $\gamma\delta$ TCR AmCyan, hCD25 BV605, and hCD56 APC. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software

daunting to delve into a 12-parameter experiment in human whole blood (Fig. 7.16). Human whole blood consists of three main cell populations—lymphocytes, monocytes (monos), and granulocytes (grannies). This figure becomes very busy and complex when you see all the combinations of cell markers used on each cell type but the point is to show you how powerful flow cytometry can be. You can separate whole blood into multiple subsets using all these markers in a single tube!

We can simplify the analysis above into a simple 3-color flow cytometry experiment by restricting our analysis on the monocytes and granulocytes. Three lineage markers can be used to identify these cells: CD14, CD11b, and CD15. Both CD14 and CD11b can be used to identify monocytes whereas CD15 is specific to granulocytes (see Fig. 7.17).

A 9-color panel can be used to subset the lymphocyte population. Starting with CD3, CD56, CD19, and gamma delta TCR, you can separate lymphocytes into NK cells, T cells, B cells, and gamma delta T cells (see Fig. 7.18). CD3⁺ T cell can be further subdivided into Resting versus Memory T cells using CCR7. You can then separate CD3⁺ T cells into CD4⁺ and CD8⁺ T cells using CD4 and CD8. CD8⁺s can be further broken down into Cytokine-induced Killers and NK T cells using CCR7 and CD56. CD4⁺s can be subdivided into Tregs using CD25. B cells can be subdivided into HLA-DR⁺ B cells and also CCR7⁺ vs. CCR7⁻ B cells where CCR7 upregulation is indicative of being recently activated. This is just the tip of the iceberg as there are many more subsets that can be isolated, but you must diversify your fluorochrome choices using some of the tandem dyes and UV/Violet fluorochromes (see Chap. 4).

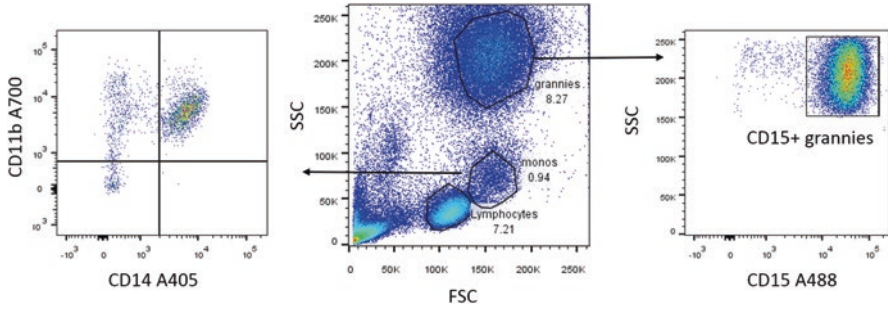


Fig. 7.17 Three-color flow cytometry on monocytes and granulocytes in human whole blood. Human whole blood was stained with the following panel of surface markers: hCD11b A700, hCD14 A405, and hCD15 A488. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software. Gating on the monocytes and granulocyte populations, CD11b, CD14, and CD15 can be used to characterize these cells

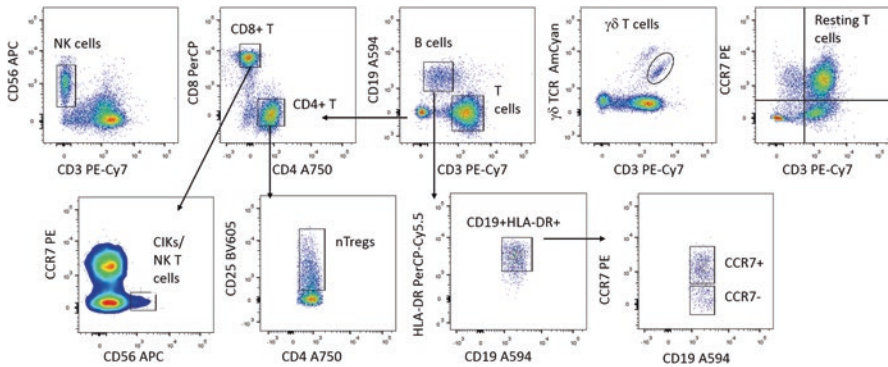


Fig. 7.18 Characterization of lymphocyte subsets in human whole blood using nine-parameter flow cytometry. Human whole blood was stained with the following panel of surface markers: hCD3 PE-Cy7, hCD4 A750, hCD8 PerCP, hCD19 A594, hHLA-DR PerCP-Cy5.5, hCCR7 PE, hγδ TCR AmCyan, hCD25 BV605, and hCD56 APC. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software®. By gating on the lymphocyte population, all the relevant T, B, NK, and γδ T cells can be identified and further subdivided

4.2 Reagents to Assess Cell Proliferation

Now we will discuss how flow cytometry can be used to label cell populations and assess functional characteristics of your cells such as proliferation. There are a couple of different ways to assess cell proliferation. One approach is to use antibodies to detect BrdU or Ki-67 (refer to Fig. 7.19a). Though both use antibodies for detection, they are different in their methodology. BrdU, an analog of thymidine, is incorporated into the cell's DNA in place of thymidine [3]. Newly synthesized DNA contains BrdU so it accumulates as the cells divide. For detection, you either have to include BrdU in your cell culture media or inject animals with BrdU if you're

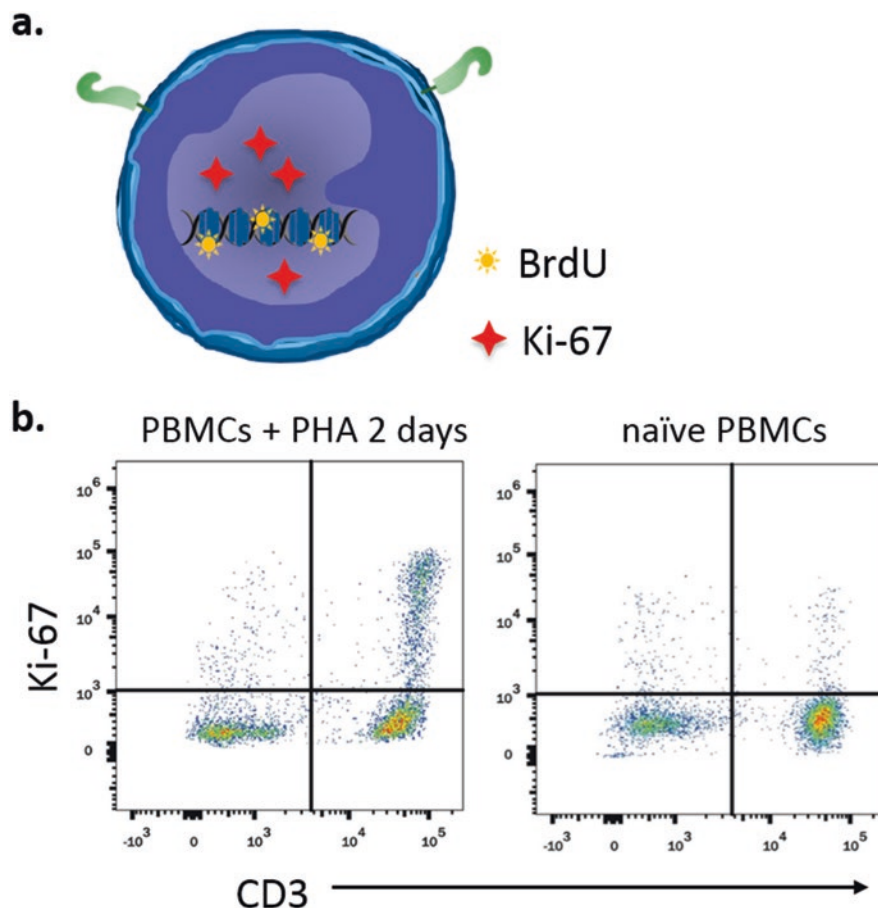


Fig. 7.19 Use of BrdU and Ki-67 to assess cell proliferation using flow cytometry. (a) Schematic showing localization of Ki-67 (nuclear, red) and BrdU (incorporates into cell's DNA, yellow) within a cell. (b) Human PBMCs were stimulated +/- PHA (5 $\mu\text{g}/\text{mL}$) for 2 days. The cells were harvested and surface stained for CD3, fixed and permeabilized using the FoxP3/transcription factor buffer set, and then stained intracellularly for Ki-67. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software. Naïve cells are shown in the right panel and PHA-stimulated cells are shown in the left panel

doing an *in vivo* assay. BrdU can then be detected via intracellular staining using a fluorochrome-conjugated BrdU antibody.

In contrast, Ki-67 is a nuclear protein that is associated with cell proliferation. It is present during all active phases of the cell cycle and is absent in resting cells. It's expression peaks during S-phase [3]. Ki-67 expression is shown above in PBMCs that have been either stimulated with PHA for 2 days (Fig. 7.19b; left dot plot) or are naïve (right dot plot). As expected, Ki-67 expression is high in the stimulated PBMCs. As evident from the data plot showing Ki-67 expression, Ki-67 accumulate as cells divide so the readout is all or none; each individual cell division cannot be visualized using this antibody-based method of detection (the same is true of BrdU).

In contrast, with cell proliferation dyes, you are able to visualize each individual cell division. This can be carried out using CFSE (which is the best known) and other proliferation dyes described below. In order to incorporate the proliferation dyes into the cells, cells are labeled for a short period of time with one of the proliferation dyes. Figure 7.20a (left panel) illustrates how these dyes work. Mechanistically, the amine-reactive dyes covalently label the cytoplasm in live cells, and as the cells divide, the dye is diluted out [4]. Each cell division can be visualized as a single peak. These proliferation dyes are available in fluorochromes across the spectrum; however, keep in mind that the fluorochrome conjugate you label your cells with should not be used on a surface or intracellular marker you are also using for analysis. Otherwise you will double stain your cells with the same fluorochrome and will have a difficult time distinguishing which population marks your proliferating cells and which is your protein of interest.

As with antibodies, there is a recommended concentration of the proliferation dye to use when labeling your cells (usually around 5 μM). However, the end user should optimize the level of labeling for their experiment. As shown in the histograms in Fig. 7.20b, we have titrated the amounts (0.1 μM –5 μM) used for labeling cells with either CFSE (which is in the FITC/A488 channel) or a Violet cell proliferation dye (which is in the A405 channel). You can see that using either dye at 5 μM results in over labeling of your cells and you end up wasting reagent. 1 μM is ideal in this experiment whereas labeling under 0.5 μM is too dim (Fig. 7.20b). Labeling with these proliferation dyes under 0.5 μM may make it difficult to visualize multiple cell divisions as they will be smashed in the first 3 decades and be very dim (Fig. 7.20a, right panel).

Cell proliferation using the violet dye can be observed in the graphs below (see Fig. 7.20c). CD3⁺ T cells were labeled with the Violet cell proliferation dye and stimulated with anti-CD3/anti-CD28 for 5 days to induce cell activation and proliferation. The far left histogram shows each cell division as a peak with the far right population representing cells that have *not* divided. Each subsequent peak to the left shows each cell division or ~ 4 cell divisions in this case. The dot plot to the right illustrates the same idea but with CD25 (a cell activation marker) on the y-axis to assess proliferation of activated T cells. There are multiple cell divisions occurring within the CD25⁺ T cell population. The same goes for the pseudocolor density plot and black and white density plot. Each cell division can be seen moving from right to left on the x-axis. This example shows that there are multiple ways to visualize cell division; either as a histogram where you can gate on a specific cell population or as a 2-parameter dot plot where you can assess cell activation at the same time.

It should also be noted that the cell proliferation dyes can be used to label cell populations for a *short* period of time such as in a killing assay (this will be discussed later in this chapter). This can be useful for tracking two different cell populations that are mixed for a period of time < 4 h. For example, NK cells can be labeled with the Violet cell proliferation dye, whereas K562 cells can be labeled with Calcein AM (a dye that is commonly used in killing assays); both populations can then be visualized on the flow cytometer.

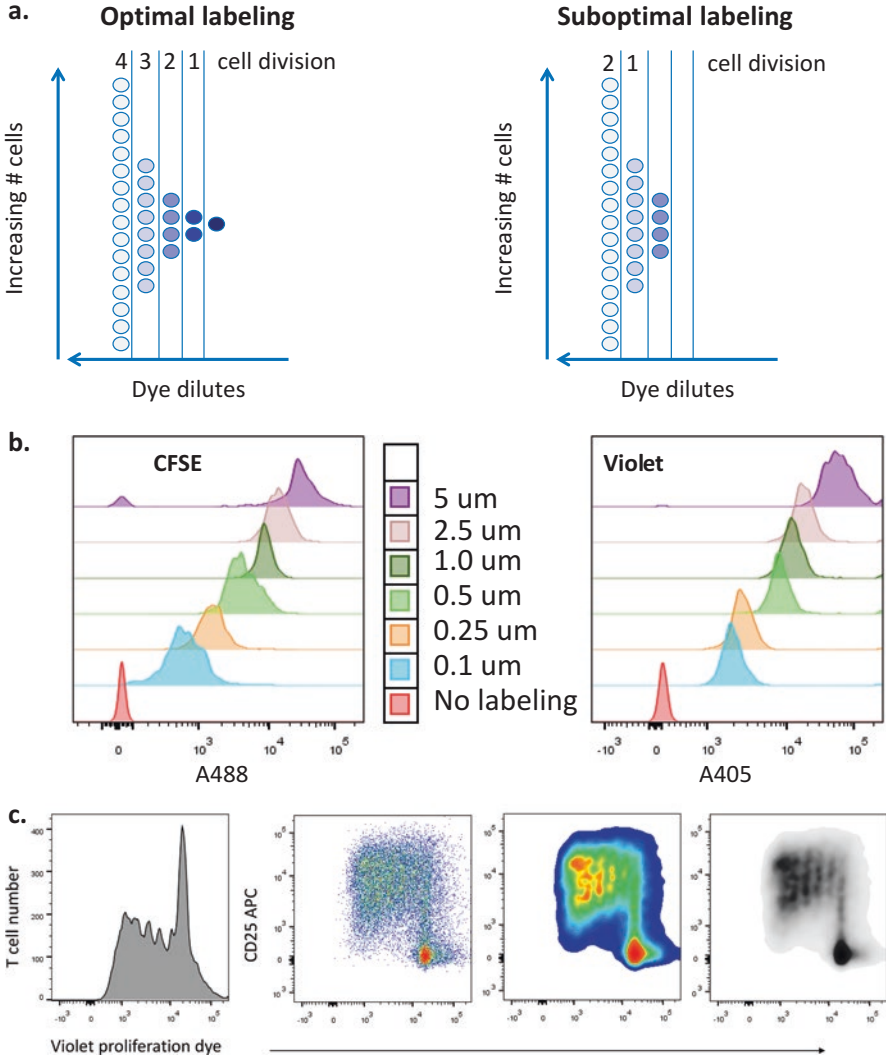


Fig. 7.20 Use of CFSE and Violet proliferation dyes for assessing cell proliferation by flow cytometry. **(a)** Schematic illustrating how the violet proliferation dye works. As cells divide, the violet cell proliferation dye dilutes out. Nondividing cells are shown on the far right, and each cell division shifts to the left. Optimal labeling of cells by the violet proliferation dye is shown in the left panel. The right panel shows suboptimal (underlabeling) labeling of cells with the violet proliferation dye where too little is being used. With underlabeling, it is difficult to see each cell division as the dye becomes too dim. **(b)** hCD3⁺ T cells were labeled with either CFSE or the violet cell proliferation dye at varying concentrations (0.1 μM –5 μM). Optimal cell labeling with both is at $\sim 1 \mu\text{M}$. **(c)** hCD3⁺ T cells were labeled with the violet cell proliferation dye (1 μM), and then placed into culture with MxhCD3 (2.5 $\mu\text{g/mL}$) and GtxhCD28 (2 $\mu\text{g/mL}$) for 5 days. Cells were harvested, and analyzed for cell proliferation and CD25 expression (using a hCD25 APC antibody). The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software. The far left histogram shows cell proliferation of T cells. The dot plots to the right show cell proliferation versus CD25 expression using pseudocolor dot plots, pseudocolor density plots, and grayscale density plots. These dot plots show proliferation of activated or CD3⁺CD25⁺ T cells

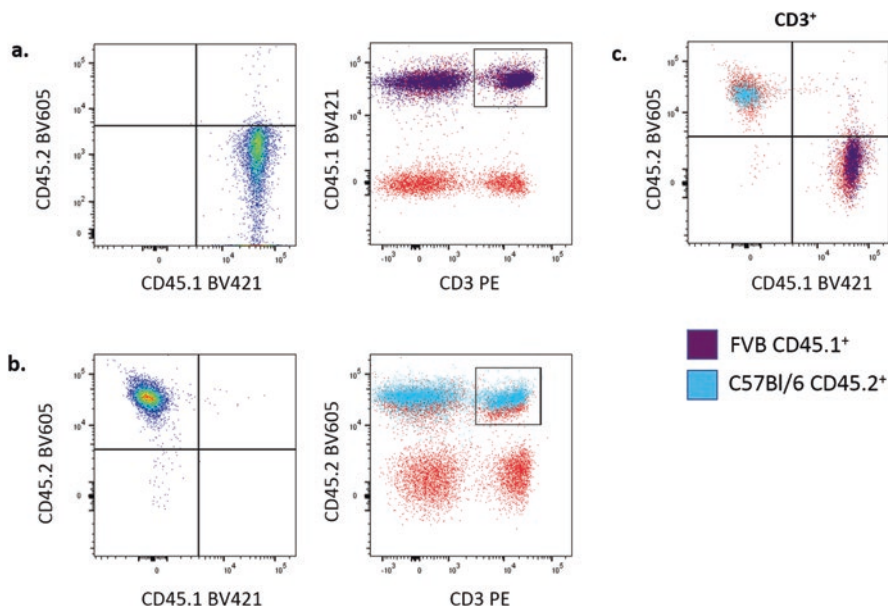


Fig. 7.21 Congenic markers for tracking cell populations in mice. C57Bl/6 and FVB mice splenocytes were mixed 1:1 and stained with mCD3 PE, mCD45.1 BV421, and mCD45.2 BV605. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software

4.3 Tracking Cell Populations in Mice Using Congenic Markers

Similar to cell tracking dyes as discussed above, congenic markers can be used to identify cell populations of interest. This is particularly useful when performing adoptive transfers in mice. For example, consider purifying CD3⁺ T cells from a C57Bl/6 mouse, and then injecting a small number into FVB mice. One way to easily identify the host and adoptively transferred T cells is by using antibodies against congenic markers. Certain mouse strains such as FVB mice express CD45.1 (Fig. 7.21a), whereas other mouse strains such as C57Bl/6 mice are CD45.2⁺ (Fig. 7.21b). Backgating can be utilized to color code CD3⁺CD45.1⁺ FVB T cells in purple (Fig. 7.21a, second panel). The same goes for the C57Bl/6 mice; CD3⁺CD45.1⁺ cells are shown in blue (Fig. 7.21b, second panel). Figure 7.21c illustrates a “mock” adoptive transfer where CD3⁺ T cells from C57Bl/6 mice have been mixed 1:1 with CD3⁺ T cells from FVB mice. Using anti-CD45.1 and anti-CD45.2, you can easily identify the CD3⁺ T cell populations from each mouse strain. Other congenic markers also exist such as Thy1.1 (CD90.1) and Thy1.2 (CD90.2). Thy1.1 is expressed in AKR/J, PL, and FVB/N mice. Thy1.2 is expressed in the following mouse strains: Balb/c, CBA/J, C3H/He, C57BL/6, DBA, NZB. Choosing the optimal congenic marker for your experiments will depend on the strain of mice you are using.

4.4 Chemokine Receptor Staining and Dependence on Temperature

Chemokine receptors are difficult proteins to develop antibodies against and even after you've developed a good antibody, you may not always be able to detect the receptor by flow cytometry. This is due to the nature of chemokine receptors. Most proteins pass through the cell membrane once, maybe twice. Because of this, the structure of these proteins changes very little. In contrast, chemokine receptors have seven transmembrane domains meaning they pass through the cell membrane seven times. Each transmembrane domain is subsequently in contact with membrane phospho-lipids which are in constant motion. This, and the presence of three intracellular and three extracellular loops that are flexible to allow for ligand binding, result in a protein that has a degree of flexibility and allows for reorganization and reorientation of its various domains. For example, compare the orientation of domains 2, 3, 4, and 7 in green in the chemokine receptor cartoon in Fig. 7.22a with the cartoon in Fig. 7.22b. Also compare the shape of the extracellular loops between domains 2 and 3 and domains 4 and 5 (in red) between the two cartoons. This flexibility, while necessary for ligand binding, can also negatively affect antibody binding. Consider a chemokine receptor-specific antibody that recognizes two separate epitopes, one on the extracellular loop between domains 2 and 3, and a second epitope on the extracellular loop between domains 4 and 5 Fig. 7.22c. The antibody would be said to bind to a conformational epitope because both epitopes need to be in the correct orientation or conformation for the antibody to bind. In the cartoon Fig. 7.22c, the domains are in the correct conformation. However, in the cartoon Fig. 7.22d, the orientation of the domains has changed leading to a change in the conformation of the extracellular loops. Thus, the chemokine receptor-specific antibody no longer binds its two epitopes.

Aside from receptor flexibility there are other factors that can compromise the detection of chemokine receptors. Much of what is known about the factors necessary to detect chemokine receptors comes from studies published by Mario Roederer's lab [5]. Chemokine receptors undergo internalization and endocytic recycling which can be induced by even subtle manipulations such as temperature shifts, media changes during Ficoll separation, and lysis of whole blood. As a consequence, receptor internalization may render the cell negative. In some cases, the addition of sodium azide prior to manipulation of cells may prevent internalization of membrane receptors; however, staining for chemokine receptors at higher temperatures such as 37 °C most often allows for maximal detection because higher temperatures (1) allow for both the receptor and cell membrane to remain fluid, increasing the likelihood that the receptors will enter the correct conformation to be recognized by receptor-specific antibodies, and (2) allow chemokine receptors that have been internalized to be recycled to the cell surface exposing the greatest number of receptors to antibodies. In comparison, staining at a more traditional temperature such as 4 °C can prevent these processes and freeze cells in a conformation that is not optimal for the detection of chemokine receptors.

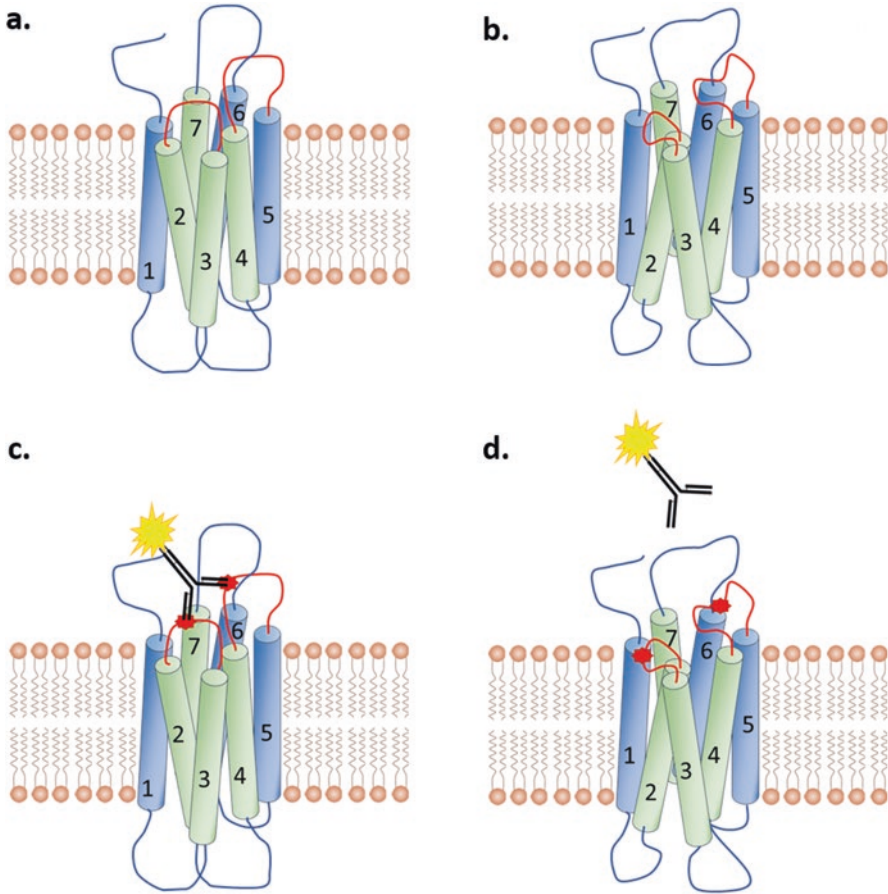


Fig. 7.22 Illustration of the flexibility of chemokine receptors and how flexibility affects the binding of chemokine-specific antibodies. Chemokine receptors are seven transmembrane receptors with three intracellular and three extracellular loops (a). Flexibility of chemokine receptor subunits can alter the orientation of the subunits and loop regions and is necessary for ligand binding (b). Chemokine receptor-specific antibodies that bind conformational epitopes between multiple loop or subunit regions (c) may not bind if the orientation of those loops and subunits are not spatially optimal or in the correct orientation (d)

To illustrate how staining temperature can affect the detection of chemokine receptors Fig. 7.23 shows the detection of CCR7 on CD4⁺ T cells on human PBMCs. When stained at 4 °C (Fig. 7.23a), CCR7 is virtually undetectable and expression does not extend beyond that of our isotype control staining by which we have set our negative gate. When stained at a slightly higher temperature (Fig. 7.23b), room temperature, we now can detect a small but significant amount of CCR7. However, when cells are stained at 37 °C (Fig. 7.23c), virtually the whole CD4⁺ T cell population now stains positive for CCR7. Not all chemokine receptors benefit from higher staining temperatures as is the case with CXCR3 on T cells (Fig. 7.23d, e). The

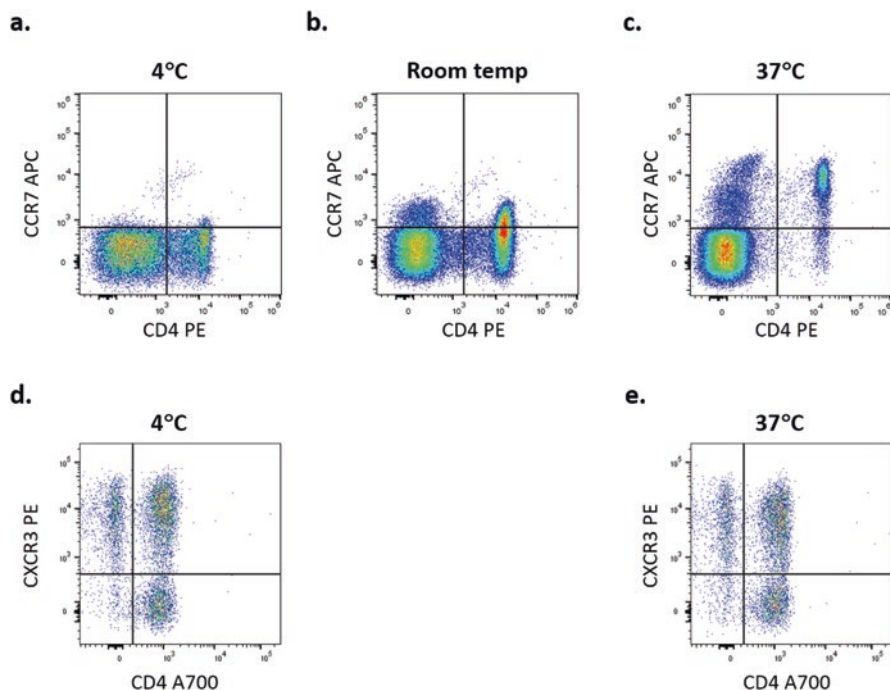


Fig. 7.23 Effects of temperature on the detection of chemokine receptors by flow cytometry. The detection of chemokine receptors by flow cytometry can be dependent on temperature. Human PBMC were stained with antibodies to hCD4 and hCCR7. When stained at 4 °C, no CCR7 can be detected on CD4⁺ T cells (a). When stained at room temperature (b), moderate expression of CCR7 can be detected. When stained at 37 °C, virtually all CD4⁺ T cells stain positive for CCR7 expression (c). The detection of CXCR3 on human T cells is not dependent on temperature as the percent and staining pattern of CXCR3 on T cells is the same whether stained at 4 °C (d) or at 37 °C (e)

percentage, distribution and MFI of CXCR3 is nearly identical on T cells whether they are stained at 4 °C (Fig. 7.23d) or at 37 °C (Fig. 7.23e).

4.5 How to Detect Degranulation in NK Cells Using a Killing Assay

NK cells are a class of innate immune cells that bridge the gap between innate and adaptive immunity [6]. We can prime NK cells by setting up a killing assay with K562 cells; the K562 cells are recognized as foreign, and the NK cells kill them. This allows for NK cell activation and we can detect NK cell degranulation by examining upregulation of the LAMP proteins. Refer to Fig. 7.24 and you can see that LAMP glycoproteins are expressed on the luminal surface of cytolytic vesicles [7, 8]. There are three LAMP proteins: CD107a is LAMP-1, CD107b is LAMP-2,

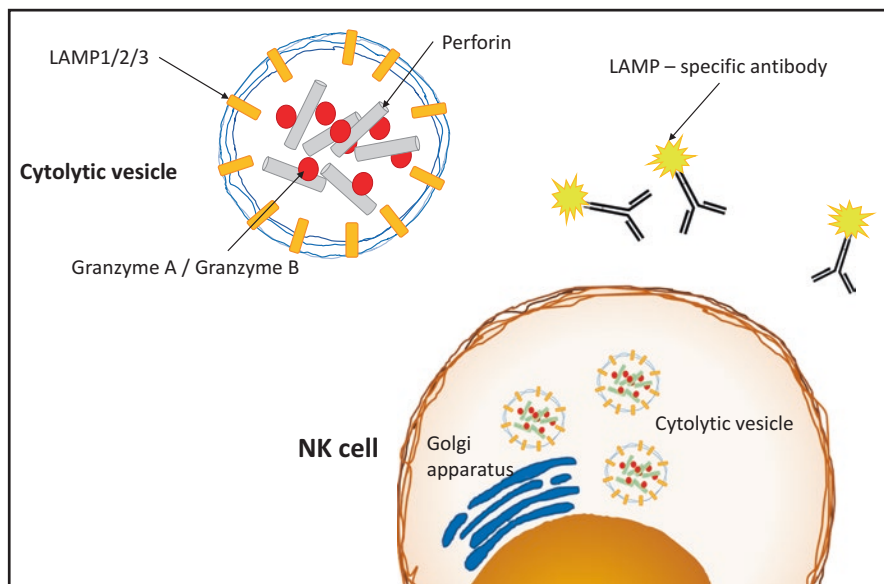


Fig. 7.24 Measuring NK cell degranulation by detecting LAMP proteins. The schematic in the upper left shows the distribution of LAMP proteins (LAMP 1/2/3), Perforin, and Granzymes A and B in cytolitic vesicles. An NK cell is depicted in the lower right with the LAMP proteins (orange cylinders) distributed in cytolitic vesicles inside the cell. During NK cell activation, the LAMP proteins, along with Perforin (gray or green cylinders) and Granzymes A and B (red circles), are upregulated so that NK cells can target foreign cells and kill them. NK cell degranulation correlates with upregulation of the LAMP proteins and can be detected using LAMP-specific antibodies (shown in yellow)

and CD63 is LAMP-3. It is important to note that these proteins are not detectable on the cell surface of resting NK cells. Upon NK activation, the proteins perforin and Granzymes A & B are also upregulated so that NK cells can perform their killing function.

During NK cell degranulation, cytolitic vesicles fuse with the plasma membrane of activated NK cells (refer to Fig. 7.25). The content of granules including granzymes and perforin is exocytosed. LAMP molecules then temporarily appear on the cell surface.

Degranulation in NK cells can be detected using LAMP-specific monoclonal antibodies. However, the LAMP antibodies must be added directly to the cell culture supernatant during the killing assay. Monensin is not required for LAMP protein detection.

Here, we are showing upregulation of LAMP1 and CD63 on NK cells during a 2-h killing assay (refer to Figs. 7.26 and 7.27). Killing assays are performed by setting up varying ratios of Effector cells (purified NK cells) with Target cells (K562 cells) which is typically abbreviated E:T (Fig. 7.26a). In order to differentiate between the NK cells and K562 cells in the killing assay, they were labeled with a Violet cell proliferation dye and Calcein AM (CAM), respectively (refer to Fig. 7.26b). CAM is a lipid green fluorescence viability dye that is readily taken up

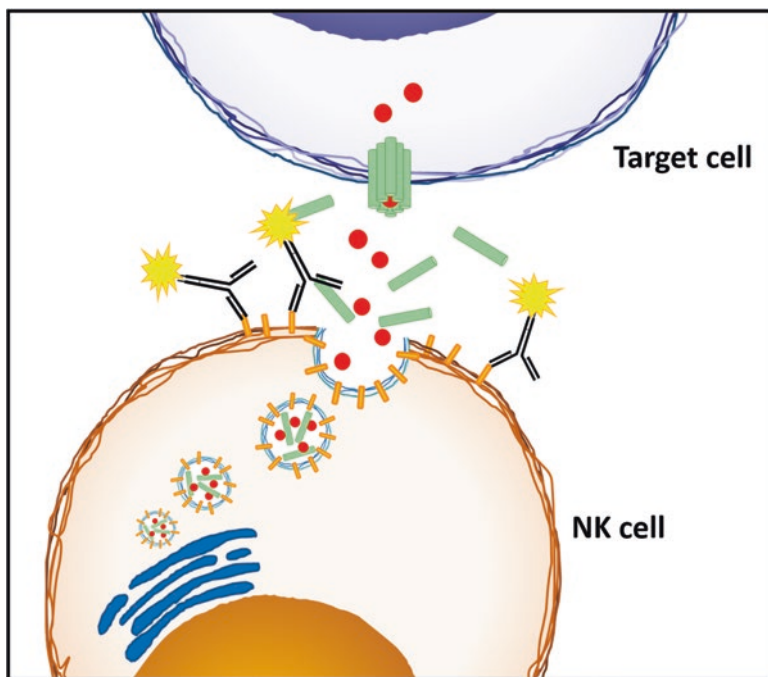


Fig. 7.25 Measuring NK cell degranulation by detecting LAMP proteins. NK cell degranulation occurs during the process of NK cells killing target cells. The schematic illustrates cytolytic vesicles fusing with the plasma membrane of activated NK cells. The content of granules including granzymes (red circles) and perforin (green cylinders) is exocytosed to kill target cells. LAMP molecules (orange cylinders) then temporarily appear on the cell surface and can be detected using LAMP-specific antibodies (shown in yellow). In order to detect LAMP expression, the LAMP antibodies need to be included in the cell culture supernatant during the killing assay

by live cells [9]. Varying ratios of Effector:Target cells were set up and are outlined below. As controls, K562 cells and NK cells were incubated alone.

1. 1:0 (violet-labeled NK cells alone)
2. 0:1 (CAM-labeled K562 cells alone)
3. 0.5:1 (violet-labeled NK cells: CAM-labeled K562 cells)
4. 5:1 (violet-labeled NK cells: CAM-labeled K562 cells)

As the CAM-labeled K562 cells die, CAM is diluted out of the cells and into the media. Refer to Fig. 7.26a to see the NK cells color-coded in red and K562 cells color-coded in blue. Figure 7.26b illustrates the percentage of K562 cells with NK cells alone (0%), K562 cells alone (99.2%), 0.5 NK:1 K562 cells (57.1%), and 5 NK:1 K562 cells (3.3%). The NK cells are effective at killing the K562 cells using a 0.5:1 ratio; compared to the control, only 57.1% remain. At the 5:1 ratio, K562 cells are virtually eliminated by the NK cells as only 3.3% remain. As mentioned above, CAM dilutes out as the cells die; this is more subtle at the 0.5:1 ratio (2 distinct CAM populations are present) and more obvious with the 5:1 ratio. Aqua dead cell exclusion dye can be used to ascertain the amount of cell death occurring in the

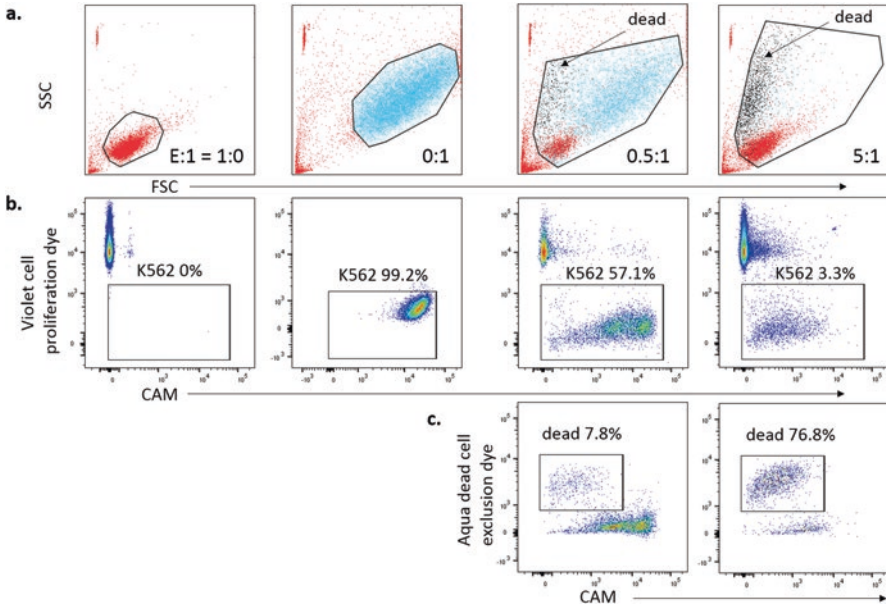


Fig. 7.26 Killing assay between preactivated NK cells and K562 cells. Human PBMCs were isolated from whole blood, followed by a 2-step purification to isolate NK cells. PBMCs were first depleted of CD14⁺ monocytes, and then NK cells were isolated using an NK cell MagCollect kit. NK cells were preactivated overnight with rhIL-12 (10 ng/mL), rhIL-15 (10 ng/mL), and rhIL-18 (50 ng/mL). The following day, the NK cells were washed, and placed into culture with rhIL-15 (10 ng/mL) for ~1 week. NK cells were labeled with a Violet cell proliferation dye (2.5 μ M) and K562 cells were labeled with CAM (0.1 μ M); both were rested at 37 $^{\circ}$ C for 30 min prior to the killing assay. The NK cells were then used in a 2 h killing assay with K562 cells at Effector (NK) to Target (K562) ratios of 1:0, 0:1, 0.5:1, and 5:1 where 1 = 0.25×10^6 cells. Isotype controls or hLAMP1 A488 and hCD63 PE were included in the cell culture media during the killing assay for detection of LAMP proteins. The cells were then harvested, stained with an Aqua dead cell exclusion dye, and surface stained for hCD56 APC and hCD3 A750. The stained cells were then run on a BD LSRFortessaTM and analyzed using FlowJo[®] software. **(a)** FSC/SSC plots of the E:T ratios described above. NK cells are circled in red, live K562 cells are shown in blue, dead K562 cells are shown in black. **(b)** Dot plots of Violet-labeled NK cells versus CAM-labeled K562 cells at the various E:T ratios are indicated in **(a)**. Percentages of CAM-labeled K562 cells are indicated. **(c)** Dot plots of CAM-labeled K562 cells versus an Aqua dead cell exclusion dye show the percentage of dead K562 cells

CAM-labeled K562 population. Figure 7.26c shows high K562 cell death (positive for Live-or-DyeTM Aqua) with the 5:1 ratio (~76.8%). By backgating on the dead K562 cells, you can also see this dead population (in black) in the FSC/SSC plots above (Fig. 7.26a).

NK degranulation is shown in Fig. 7.27. The dot plots on the left show low/negative levels of LAMP1 and CD63 expression on CD56⁺ NK cells alone, as expected. LAMP proteins are not induced on the surface on resting NK cells. However, with an E:T ratio of 5:1, NK cell degranulation is taking place as both LAMP1 and CD63 are upregulated. This supports the data shown in Fig. 7.26 as the NK cells have killed most of the K562 cells at the 5:1 ratio. In addition to detecting proliferation

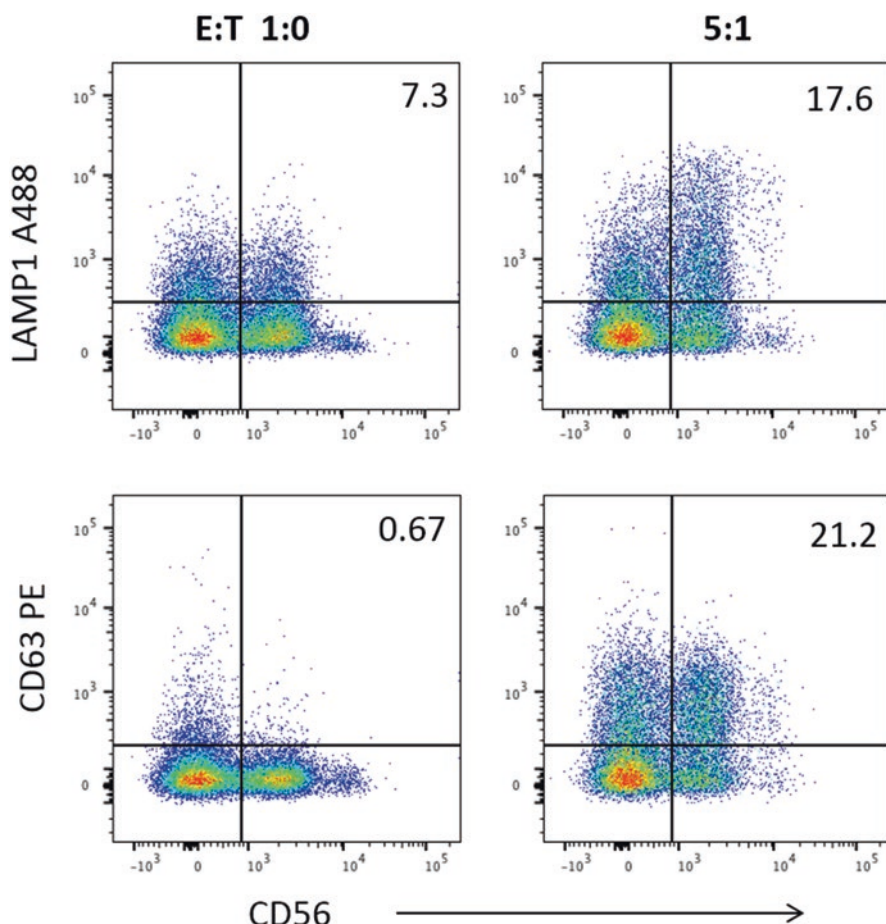


Fig. 7.27 LAMP1 and CD63 are upregulated during a 2 h killing assay between NK cells and K562 cells. Refer to Fig. 7.26 for details on how the killing assay was set up. In this experiment, NK cells and K562 cells were not labeled with the violet proliferation dye or CAM, respectively. Isotype controls or hLAMP1 A448 and hCD63 PE were included in the supernatant during the killing assay to assess NK cell degranulation. The cells were harvested, stained, and run on the flow cytometer as described above. Basal levels of LAMP1 and CD63 are shown in the left panels (E:T is 1:0). Upregulation of LAMP1 and CD63 is shown in the right panels using an E:T ratio of 5:1

and cell death, this experiment shows that flow cytometry can be utilized to examine cellular functions such as degranulation and killing.

4.6 Protein Detection Using Fluorokines

Fluorokines were developed as a tool to allow researchers to detect cell surface cytokine receptors using a flow cytometer. Much like antibodies, these ligands can be conjugated to different fluorochromes and then cells can be stained with these

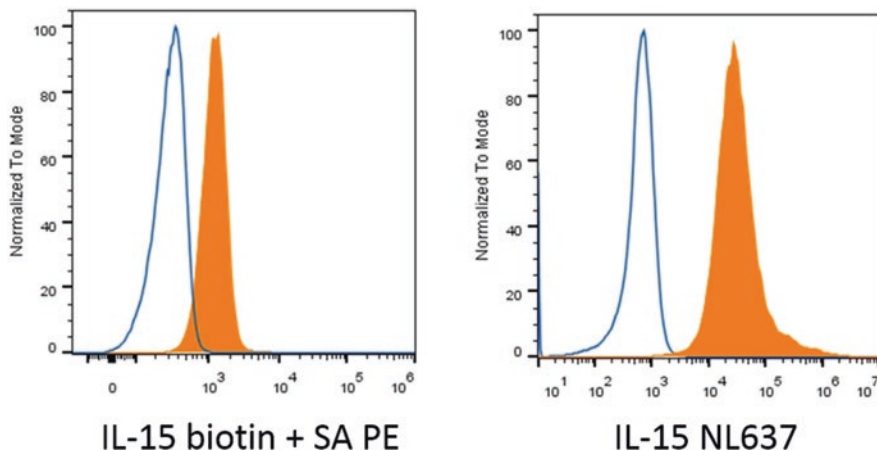


Fig. 7.28 rhIL15 expression on TF-1 cells using Fluorokines. TF-1 cells were stained with rhIL15 either as a biotinylated protein or NL637-conjugated protein. rhIL15 biotin was detected using Streptavidin PE. The stained cells were then run on an Acea Novocyte™ and analyzed using FlowJo® software

conjugated proteins. Furthermore, for many of the proteins, a blocking antibody can be used to block binding of the receptor to various cells. Although useful, optimization is always needed for these fluorokines, which can be influenced to varying degrees by external conditions. Shown below in Fig. 7.28 are examples of rhIL15 conjugated to biotin (detected with Streptavidin PE) and or directly conjugated to NL637, stained on TF-1 cells. Controls are unstained cells. This data illustrates that biotinylated and directly conjugated fluorokines are easily detected like antibodies and can be used to assess cellular function.

4.7 Dump Channels

Most often when doing a multi-color flow cytometry experiment we identify and gate on cells based on their expression of unique markers such as CD3, CD19, CD14, but sometimes we are interested in cells that don't express any one marker that is truly unique to them but are rather defined by a combination of several markers. Many times, these are small populations of cells that are nearly invisible and get lost in the vast numbers of cells of a similar size and granularity. A good example of this is illustrated with myeloid dendritic cells that can be found in human blood. These cells are typically characterized by, among others, the expression of HLA-DR, the myeloid antigens CD11b, CD11c, CD13, CD33, which are common to all myeloid cells, and combinations of CD141 (Thrombomodulin), CD1c, CD1d, and CD16 [10, 11]. None of these markers are truly unique to myeloid dendritic cells as they are all expressed other cell types making it necessary to exclude those other cell types to limit analysis to just myeloid dendritic cells. Moreover, myeloid

dendritic cells do not possess a unique forward and side scatter profile, being found in the lymphocyte gate along with T cells, B cells, NK cells, and plasmacytoid dendritic cells. However, these cells lack the expression of CD3 (T cells), CD19 (B cells), CD14 (monocytes), and CD56 (NK cells).

Since CD3, CD19, CD14, and CD56 are traditional lineage markers for T cells, B cells, NK cells and monocytes, blood dendritic cells would be considered “lineage negative” (“lin-negative”) cells. This means that once these markers and the cells that express them have been excluded, the remaining cells will be significantly enriched for myeloid dendritic cells. This is where a “dump” channel becomes effective. Since we are not interested in identifying cells that expresses CD3, CD14, CD19, and CD56, we don’t care if all of those markers are on the same color. Rather than waste four colors on markers we aren’t interested in, we will “dump” all of these markers on one color. Usually, this is a relatively dim color such as FITC, A700, or A750 since CD3, CD14, CD19, and CD56 are highly expressed on T cell, monocytes, B cells, and NK cells so even a dim fluorochrome will provide adequate discrimination of those populations without sacrificing a bright color which will be important for phenotyping markers on myeloid dendritic cells which aren’t highly expressed. Dump channels are not limited to lineage markers but tend to most commonly use combinations of lineage markers.

To illustrate the use of a dump channel, in Fig. 7.29 we are showing the detection of myeloid dendritic cells in human whole blood. Myeloid dendritic cells are found within the lymphocyte compartment, so a broad gate is drawn around the lymphocyte population and into the lower monocyte population (Fig. 7.29a). The dump channel we have assembled consists of A405-conjugated antibodies to CD3, CD14, and CD19. We have purposely left out an antibody to CD56 to provide us with an A405-negative population. Myeloid dendritic cells express HLA-DR but do not express CD3, CD14, or CD19 so we will plot the lymphocytes with HLA-DR on the y-axis and the CD3/14/19 A405 dump channel along the x-axis (Fig. 7.29b). This results in five discernable populations (counterclockwise from bottom left): a population of NK cells (HLA-DR⁻CD3/14/19 dump⁻); a population of T cells (HLA-DR⁻CD3/14/19 dump⁺); a small population of monocytes since we’ve excluded most of the monocyte population when drawing our lymphocyte gate (HLA-DR^{mid}CD3/14/19 dump⁺); a population of B cells (HLA-DR^{hi}CD3/14/19 dump⁺); and a small population of blood dendritic cells (HLA-DR^{hi}CD3/14/19 dump⁻). You can get an idea of how rare this population is compared to the other major cell subsets found in the lymphocyte gate. A gate is drawn around the population of HLA-DR^{hi}CD3/14/19 dump⁻ blood dendritic cells that are then separated into plasmacytoid versus myeloid dendritic cells by the expression of CD11c and CD304/BDCA-4 (Fig. 7.29c). Myeloid dendritic cells can further be divided into CD1d⁺ and CD16⁺ subsets (Fig. 7.29d) whereas plasmacytoid dendritic cells express neither marker (Fig. 7.29e). Without first excluding T cells, B cells, and monocytes with a lineage dump channel, identifying myeloid dendritic cells would have been much more difficult.

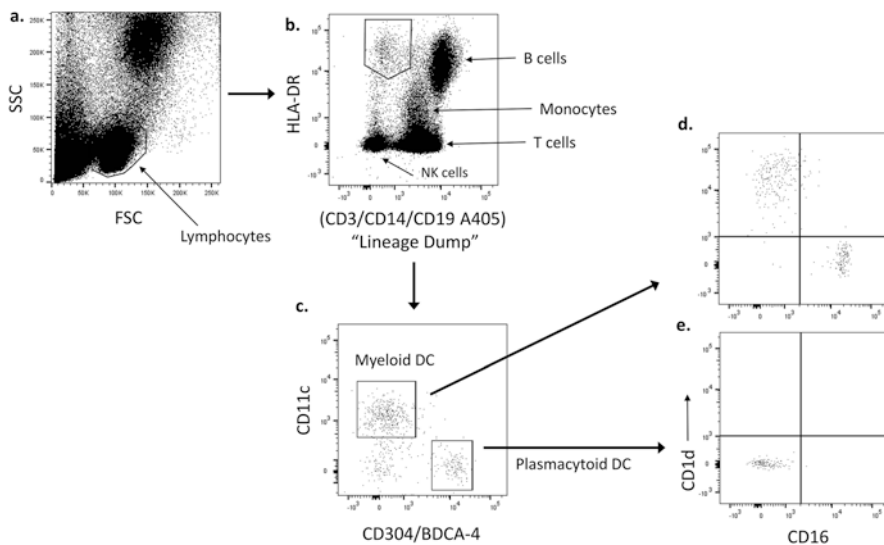


Fig. 7.29 Using a dump channel to identify human blood dendritic cells. Human blood dendritic cells are a minor cell population within the lymphocyte population. Lymphocytes were gated (a) and then blood dendritic cells were identified as HLA-DR⁺CD3⁻CD14⁻CD19⁻ cells (b). To facilitate the detection of blood dendritic cells a “lineage dump channel” was created containing the lineage markers CD3, CD14, and CD19 all on A405. HLA-DR⁺Lineage⁻ cells were further separated into Myeloid DC and Plasmacytoid DC based on the expression of CD11c and CD304/BDCA-4 (c) and the expression of CD1d and CD16 (d and e)

5 Intracellular Staining Considerations

5.1 Intracellular Staining Buffers

The purpose of intracellular staining is to detect proteins that are nuclear such as transcription factors, and proteins that accumulate inside the cell such as cytokines. Detailed protocols for intracellular staining can be found in Chap. 9. Intracellular staining requires two steps in most cases (there are exceptions) and these include a Fix step and a Permeabilization step. The three main Intracellular staining buffer systems are listed below:

1. Formaldehyde and Saponin that can be used to detect secreted cytokines.
2. FoxP3/transcription buffer system that is used to detect transcription factors but also works for secreted cytokines.
3. Formaldehyde and Methanol that is used for detection of phospho proteins.

Now we will discuss the different components available for the Fix and Permeabilization buffers. For the Fix buffers, there are two main ones used: Formaldehyde and Glutaraldehyde. Formaldehyde has two derivatives: Formalin and Paraformaldehyde. Formalin is simply a saturated 37% Formaldehyde solution,

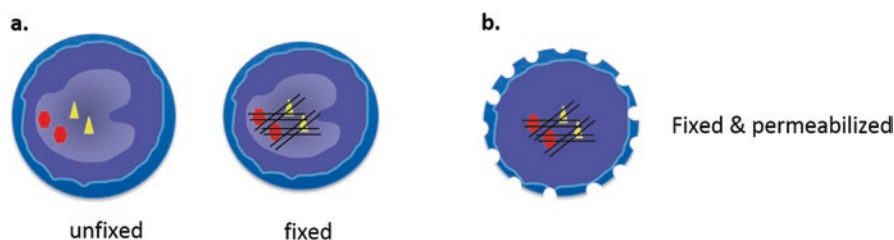


Fig. 7.30 Fixation and Permeabilization of cells. The schematic illustrates what happens once cells are fixed (**a**) and permeabilized (**b**). (**a**) During fixation, proteins (shown in red and yellow) inside the nucleus become cross-linked to stabilize them. (**b**) After fixation, the process of permeabilization creates holes in the cell membrane by various mechanisms (depending on the permeabilization buffer used) to allow access of the intracellular antibodies

whereas Paraformaldehyde (or PFA) is polymerized formaldehyde. 10% Formalin is roughly equivalent to 4% Formaldehyde [12]. It is not uncommon for Formaldehyde and PFA to be used interchangeably but they are slightly different. Formaldehyde is solution-based and Paraformaldehyde is prepared from a powder. Mechanistically, they function the same and the percentages used for either are interchangeable. Another known fixative is Glutaraldehyde but is not recommended as it produces high background staining and poor antibody staining; therefore, we won't go into any more detail on this fixative in this book. To illustrate how fix buffers work, refer to Fig. 7.30a. The cell on the left is unfixed; with a fixative is applied such as Formaldehyde, the proteins become cross-linked and “fixed” in the cell on the right. The cell also shrinks a little in size due to fixation. Formaldehyde fixation freezes cellular processes by cross-linking proteins to one another, creating a lattice. Fixation also prevents further signaling and stabilizes the cell structure [13].

There are three options for Permeabilization buffers. They include Saponin, the alcohol-based permeabilization buffers, and detergent-based permeabilization buffers. *Saponin* is an amphipathic glycoside that can be derived from plants and marine organisms such as sea cucumber. Low percentages are used to permeabilize cells. Saponin interacts with membrane cholesterol and removes it, thereby leaving holes in the cell membrane [14].

For the *alcohol-based permeabilization buffers*, researchers commonly use Methanol, Acetone, and Ethanol. These alcohol-based permeabilization buffers are also known as solvents and permeabilize cells by dissolving lipids in the cell membrane [14]. It is important to fix your cells prior to permeabilization with Methanol as Formaldehyde fixation prevents the clumping that results when cells are exposed to alcohols [13]. In this book, we'll show some examples of phospho-staining using Formaldehyde & Methanol in subsequent chapters. Methanol, along with acetone and ethanol, are typically used at a concentration of 90%. Because of their harsh nature, you have to be careful using these with certain fluorochromes, especially if surface staining must be carried out first.

The third class of permeabilization buffers are the *detergent-based* ones such as Triton X-100. These detergents function by removing lipids but are not selective and may also remove proteins as well [14]. These are typically used at very low concentrations or 0.1–0.5%. After a cell is fixed, a permeabilization buffer is used

to complete the intracellular staining process before an antibody requiring intracellular staining is added. The permeabilization buffers create holes in the cell membrane so that antibodies can gain access to their respective receptors and bind (refer to Fig. 7.30b). Specifically, intracellular cytokines such as IFN γ reside within the nucleocytoplasm, and phospho-proteins and transcription factors reside within the nucleus. All are intracellular meaning the antibody of interest can't bind unless it is able to get inside the cell.

5.2 *Detection of Secreted Cytokines*

As mentioned above, secreted cytokines can be detected using intracellular staining; however, additional steps are required. Specifically, either Monensin or Brefeldin A must be added to your cell culture prior to intracellular staining to allow the cytokines of interest to accumulate inside the cell. It should be noted that phospho-proteins or transcription factors do not require Monensin or Brefeldin A as they are not secreted. In addition, depending on your cell type, reactivation with strong stimuli such as PMA and Ionomycin for 3–5 h, can help boost cytokine secretion. This is the case with T cells but is not necessary with other cell types such as monocytes. Another type of stimuli that can be used are ones that are antigen-specific such as a peptide antigen. For example, using a system in which mice have been immunized with influenza virus, once you harvest the cells of interest, you can pulse or incubate those cells *ex vivo* with flu peptide to optimally activate the flu-virus-specific T cells. These cells can then be used for further analysis.

We mentioned above that Monensin is required to allow for secreted cytokines to accumulate inside the cell. Monensin, which is a Golgi stop, and Brefeldin A, which is a Golgi plug, function as protein transport inhibitors to allow for these secreted cytokines to accumulate in the cell. Generally speaking, Monensin and Brefeldin A are typically added to your cell culture for 3–5 h prior to Intracellular staining. If reactivation is required with PMA and Ionomycin, Monensin or Brefeldin A can be included at the same time for 3–5 h prior to intracellular staining. To illustrate how these protein transport inhibitors work, refer to Fig. 7.31 [15] where we are showing IFN γ protein inside the cell. Without Monensin or Brefeldin A present, IFN γ would normally be exported out of the cell going through the Endoplasmic Reticulum, and Golgi apparatus as shown.

We won't go into gross detail with the mechanism of action of Brefeldin A or Monensin as it is beyond the scope of this book but will briefly describe how they work. In this schematic (Fig. 7.31), we show that Brefeldin A prevents IFN γ secretion by redistributing intracellularly produced proteins from the cis/medial Golgi complex to the endoplasmic reticulum (the blue circles represent vesicles). Alternatively, Monensin prevents protein secretion by interacting with the Golgi transmembrane Na⁺/H⁺ transport system. Monensin is a Na⁺/H⁺ ionophore where it facilitates the exchange of sodium ions for protons. One consequence of this exchange is the neutralization of acidic intracellular components within the cell such as the trans Golgi apparatus cisternae and associated lysosomes and endo-

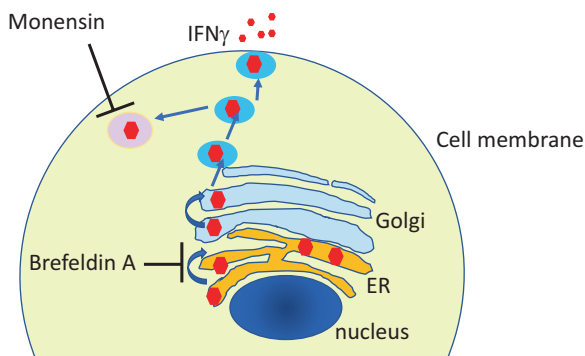


Fig. 7.31 Reactivation of cells with Monensin or Brefeldin A for the detection of IFN γ secretion. The schematic shows two different mechanisms by which IFN γ (red hexagon) stays trapped inside the cell for detection using an IFN γ -specific antibody by flow cytometry. Brefeldin A prevents IFN γ secretion by redistributing IFN γ from the Golgi complex (light blue) to the endoplasmic reticulum (orange). Monensin blocks IFN γ export by interacting with the Golgi transmembrane Na⁺/H⁺ transport system. IFN γ remains trapped in a lysosome (indicated by the purple circle). In the absence of either Brefeldin A or Monensin, normal export of IFN γ outside the cell is accomplished via vesicles (blue). Refer to the text for more details

somes. The net effect is altering the pH of the intracellular cellular components [16, 17]. IFN γ is shown in a lysosome indicated by the purple circle. Thus, with either of these protein transport inhibitors, IFN γ stays trapped inside the cell for optimal detection using an IFN γ flow antibody.

It is absolutely critical to include PMA, Ionomycin, and Monensin in your cell culture prior to intracellular staining; otherwise, you won't be able to detect your secreted cytokine(s) of interest. The middle panel in Fig. 7.32 shows hTh1 cells stimulated with Monensin only; there is no detectable IFN γ , even in a Th1 cell that produces a high amount of IFN γ . In contrast, when you stimulate with the Tocris reactivation cocktail containing PMA, Ionomycin, and Monensin, you can see high levels of IFN γ (far right dot plot). However, one drawback with this reactivation cocktail is that PMA lowers CD4 expression on T cells. Cells restimulated with PMA, Ionomycin, and Monensin essentially lose good separation between CD4⁺ and CD4⁻ populations (red ellipse, Fig. 7.32, far right panels); they become bunched together and have a lower MFI. CD4 is a well-known activating coreceptor, and reactivation of T cells with strong stimuli such as PMA causes CD4 receptor recycling/internalization due to down-stream signaling cascade activation [18]. Foster et al. recommend staining with the CD4 antibody after fixation/permeabilization and including it with the intracellular antibody/antibodies stain [17].

5.3 Optimizing Fixation, Staining Order, and Using the Correct Intracellular Buffer System

Now that you are well versed in the intracellular staining buffer systems that are available, and the importance of including protein transport inhibitors when detecting secreted cytokines, we'll go over how to optimize intracellular staining. First,

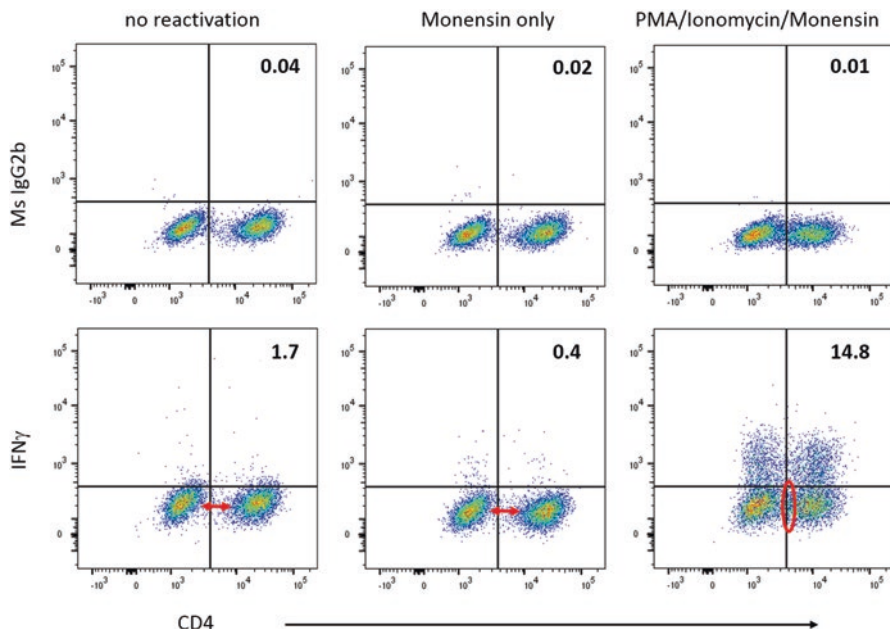


Fig. 7.32 Reactivation with PMA, Ionomycin, and Monensin are required for detection of IFN γ in hTh1 cells. Human CD3⁺ T cells were purified and then cultured for 5 days in the presence of Th1 promoting stimuli (MxhCD3 (2.5 μ g/mL), GtxhCD28 (2 μ g/mL), and rhIL-12 (10 ng/mL)). Prior to intracellular staining, cells were (1) not reactivated (left panels), (2) reactivated with Monensin only (3 μ M; middle panels), or (3) reactivated with PMA, Ionomycin, and Monensin (Tocris Cell Activation cocktail; right panels) for 3 h. After harvesting the cells, cells were surface stained with hCD4, fixed (1% Formaldehyde) and permeabilized (1 $\times$ Saponin), then stained intracellularly for either Mouse IgG2b or hIFN γ

we'll discuss the importance of choosing the correct fixation percentage. Next, we'll go over how staining order can influence detection of surface markers. Finally, we'll make the point that not all intracellular staining Fix/Permeabilization buffers are created equal; some intracellular proteins such as transcription factors and phospho-proteins required specialized intracellular staining buffer systems.

5.3.1 Optimizing Fixation Percentage

In this section, we illustrate that using too high a concentration of fix buffer is not ideal. In order to induce IFN γ secretion, we stimulated resting CD3⁺ T cells with the Tocris reactivation cocktail for 3 h. The Tocris cocktail contains PMA, Ionomycin, and Monensin. We then blocked our cells for Fc receptors, and carried out surface staining for CD45RA A488. CD45RA can be used to distinguish naive and memory T cells, where CD45RA⁺ cells are naive, and CD45RA⁻ cells are memory T cells. After surface staining, we fixed the cells with either 1%, 2%, or 4% Formaldehyde, followed by 1 $\times$ Saponin or SAP as our permeabilization buffer (refer to Fig. 7.33).

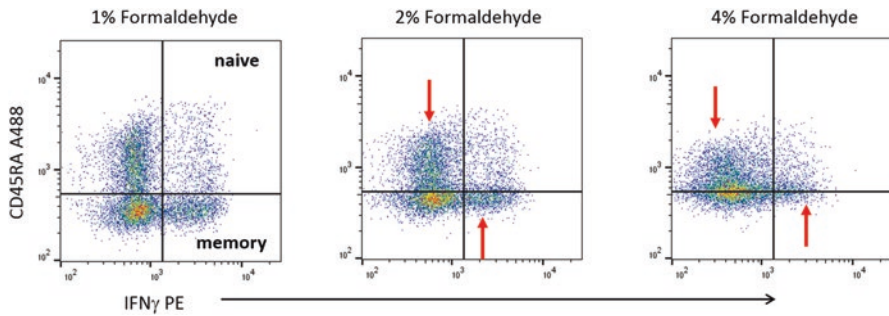


Fig. 7.33 Optimizing fixation in human Memory T cells: less is more. Resting hCD3⁺ T cells were stimulated with the Tocris reactivation cocktail for 3 h. The cells were surface stained for hCD45RA A488. After surface staining, the cells were fixed with 1%, 2%, or 4% Formaldehyde, followed by permeabilization with 1× Saponin. Cells were then stained intracellularly for hIFN γ PE (gates were set using isotype controls; data not shown). The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software

As you can see, 1% Formaldehyde is ideal. Your staining for IFN γ PE is distinct, as well as the expression of the surface marker, CD45RA A488. You can see that IFN γ is primarily produced by memory T cells or CD45RA⁻ cells. Once you start using 2% Formaldehyde, IFN γ negative and positive populations become harder to distinguish and CD45RA expression dims. 4% Formaldehyde is clearly the worst as shown on the far right dot plot. IFN γ expression is poorly defined and CD45RA is really smashed into the lower left quadrant. In a nutshell, less is more with fix buffer concentrations. In data not shown, the FoxP3 buffers are also compatible when detecting IFN γ and CD45RA expression.

5.3.2 Staining Order Matters When Detecting Both Surface and Intracellular Markers

In order to optimally assess intracellular and surface markers together, it is important to consider the staining order. Surface markers can get dimmed and smashed together with fixation. So some researchers prefer to stain their surface markers first to ensure they are bright. Others like to fix and permeabilize their cells first to preserve expression of their intracellular marker. Fixation can also dim endogenous fluorescence so this can lower background staining. Either approach has its advantages. In this example, we are using the FoxP3/Transcription factor buffer system on human PBMCs (refer to Fig. 7.34). The top panel of dot plots shows the optimal staining order. Stain with surface markers 1st, and then fix/permeabilize. As a result, HLA-DR PerCP, CD25 A700, and CD4 A405 have nicely separated and distinct cell populations.

In the bottom dot plots, we are showing the result of fixing and permeabilizing your cells first, followed by intracellular and surface marker staining together. In contrast to the dot plots above, the expression pattern of CD25 A700 is smashed. In addition, CD4 A405 separation is poor as the negative and positive populations are bunched together. In this scenario, the surface markers are dimmed and smashed/

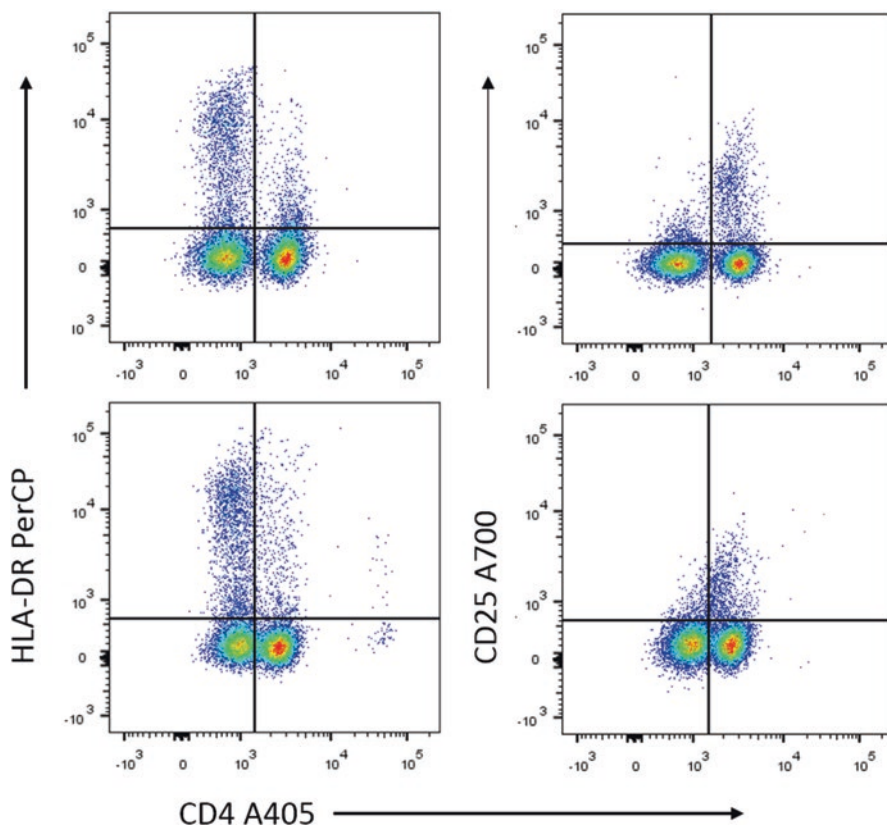


Fig. 7.34 Surface and Intracellular staining: order matters. Human PBMCs were stained for hCD4 A405, hCD25 A700, and hHLA-DR PerCP either first as surface stains (top panels) or after fixation and permeabilization with the FoxP3/Transcription factor buffer set (bottom panels). Gates were set using isotype controls (data not shown). The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software

poorly separated. Now you'll notice that HLA-DR PerCP staining was not compromised with fixation so not all surface antigens and fluorochromes will be affected the same way. If possible though, the safe bet is to surface stain 1st, and then carry out Intracellular staining. The exception occurs when reactivating cells with PMA, Ionomycin, and Monensin. Some surface markers get internalized with reactivation, and detection can be better when those markers are stained after fixation/permeabilization and during the incubation with the intracellular antibodies [17].

5.3.3 Intracellular Buffer Systems for Transcription Factors and Phospho-Proteins

As we mentioned above, not all intracellular buffer systems are compatible with every type of intracellular marker. Detection of secreted cytokines is not picky as you can use either the Formaldehyde/SAP or FoxP3/Transcription factor buffer

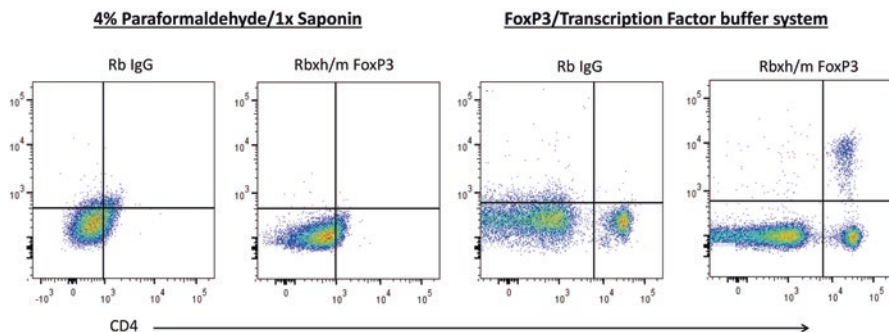


Fig. 7.35 Optimal Intracellular staining buffer system for Detection of FoxP3. Human PBMCs were placed into culture for 2 days with hIL-2 (20 ng/mL) and hTGF- β (10 ng/mL) to induce Treg expansion (iTregs). Cells were harvested, and stained with hCD4 and Rabbit (Rb) IgG or h/mFoxP3. Intracellular staining was carried out using 1) 4% Paraformaldehyde/1 $\times$ Saponin (left panels) or 2) the FoxP3/Transcription factor buffer system (right panels). Cells were then acquired on a BD LSRFortessaTM and analyzed using FlowJo[®] software. In the 2 left panels, CD4 was stained intracellularly along with Rb IgG or FoxP3 using a suboptimal buffer set (4% Paraformaldehyde/1 $\times$ Saponin). In the right 2 panels, surface staining for CD4 was carried out first, and then intracellular staining for Rb IgG or FoxP3 was carried out after fixation and permeabilization using the optimal buffer set (FoxP3/Transcription factor buffer system)

system. In this section, we will demonstrate that this is not the case with transcription factors and phospho-proteins; they require specialized Fix/Permeabilization buffers for optimal detection.

Detection of Transcription Factors: FoxP3

FoxP3 is a transcription factor specific to Tregs. It is clear that using the 4% PFA/1 $\times$ Saponin combination buffer system does not allow for good detection of FoxP3 in human iTregs—you really can't see it at all as shown in the left dot plots (Fig. 7.35). CD4 expression also is suboptimal due to the high percentage of PFA used, and carrying out surface staining after fixation/permeabilization. However, the FoxP3/Transcription factor buffer system allows for optimal detection of the FoxP3 antibody (Fig. 7.35, right dot plots). In addition, CD4 negative and positive populations are clearly defined as surface staining was done first. This example shows that some antibodies must be used with a specific fix and permeabilization buffer system so they can be detected. In general, researchers have found that addition of the nonionic detergents (such as Saponin and or Tween) during both the fixation and subsequent permeabilization steps allowed for the optimal detection of transcription factors [19].

Detection of Phospho-Proteins: Phospho-Stat1

While paraformaldehyde and saponin or the FoxP3 buffer system works well for many intracellular proteins and transcription factors, the detection of phosphorylated proteins requires a slightly different protocol. In 2003, Peter Krutzik and Gary Nolan

examined ten different cellular fixation and permeabilization techniques to determine which combination was effective in detecting phospho-specific proteins and found that between 1–10 min of paraformaldehyde fixation followed by 10 min of methanol treatment provided optimal results [13]. Ethanol and acetone can also be used for phosphorylated proteins following fixation with 1% Formaldehyde but methanol outperformed both. One advantage to methanol permeabilization is that the cells can be stored for an extended period of time (at least 5 weeks) at –20 °C to –80 °C prior to staining. It is not known exactly why methanol is particularly effective in detecting phosphorylated proteins, but it has been suggested that through its dehydrating effects, methanol may lead to some protein denaturation and that many phospho-specific antibodies are often generated against linear (or denatured) peptides.

To demonstrate this, human monocytes were stimulated with IFN γ , then fixed with 1% Formaldehyde and permeabilized with methanol before being stained intracellularly with a phospho-specific Stat 1 antibody (Fig. 7.36a). Unstimulated monocytes are represented by the blue histogram which show a comparable level of phosphorylated Stat1 staining compared to background isotype control staining represented by the red histogram. By comparison, IFN γ stimulation has resulted in an

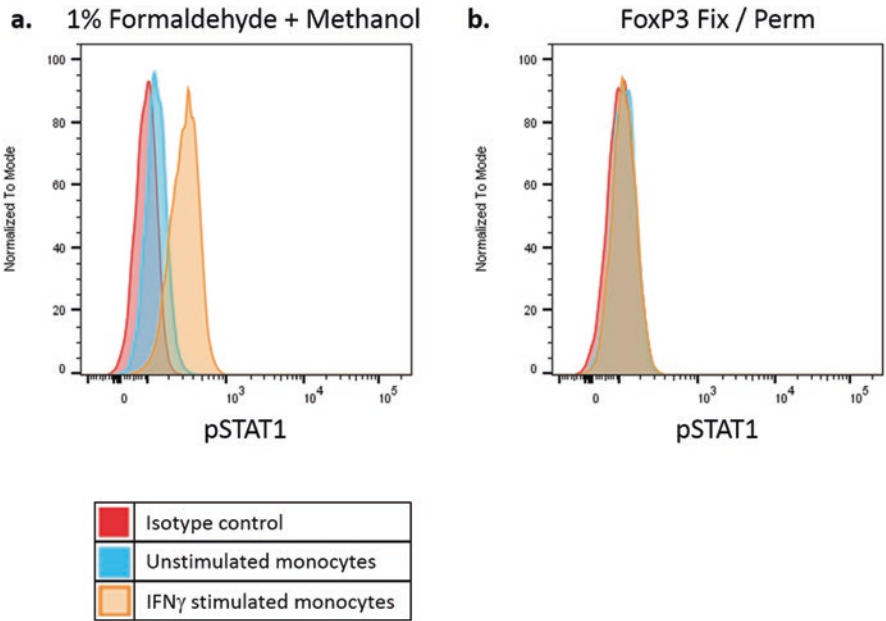


Fig. 7.36 The effects of cell permeabilization with methanol on the detection of intracellular phospho-proteins. Groups of human monocytes were serum starved in RPMI-based media lacking serum for 30 min at 37 °C. One group of PBMCs was stimulated with 50 ng/mL IFN γ + 50 ng/mL LPS for 20 min (orange histograms) while a second group remained unstimulated (blue histograms). Isotype control staining is represented by the red histograms. PBMCs from both groups were fixed with 1% paraformaldehyde and then further divided into two groups. One group was permeabilized with methanol (**a**) while the second group was permeabilized with FoxP3 permeabilization buffer (**b**) before the addition of an antibody to detection phospho-STAT1

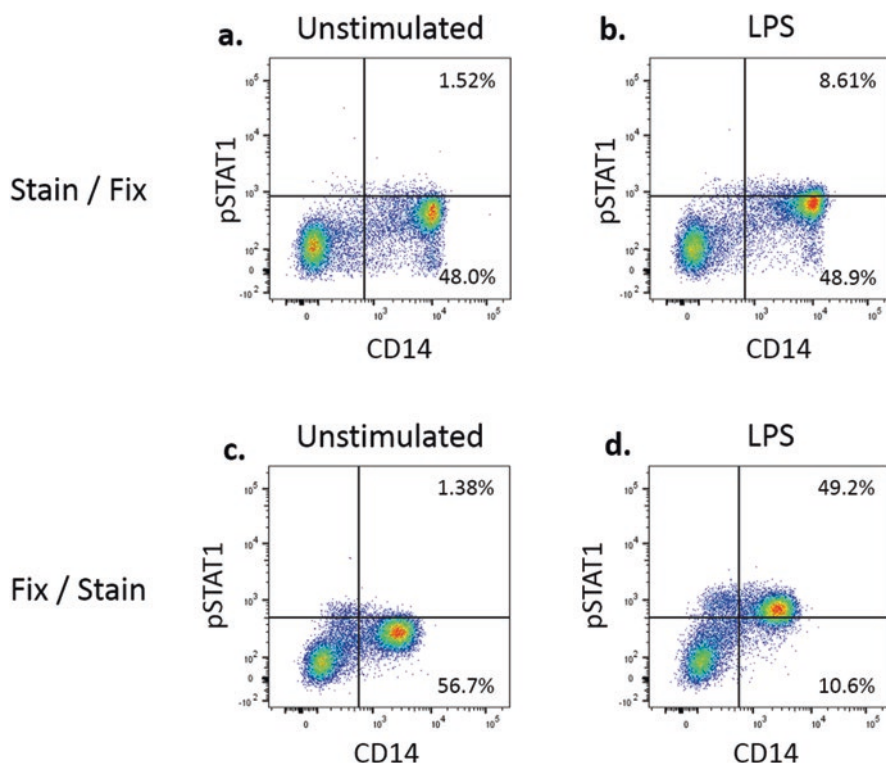


Fig. 7.37 The effects of Stain and Fix order on the detection of phospho-proteins. Groups of human PBMCs were serum starved in RPMI-based media lacking serum for 30 min at 37 °C. One group of PBMCs was stimulated with 50 ng/mL IFN γ + 50 ng/mL LPS for 20 min (**b** and **d**) while a second group remained unstimulated (**a** and **c**). PBMCs from both groups were then further divided into two groups. One group was immediately fixed with 1% paraformaldehyde (**c** and **d**) while the second group was stained with a hCD14 FITC antibody prior to fixation with paraformaldehyde (**a** and **b**). Cells were fixed for 10 min at room temperature before permeabilizing with methanol and staining for phospho-STAT1. Cells that were immediately fixed after IFN γ and LPS stimulation (**c** and **d**) were stained for CD14 concurrently with phospho-STAT1

increase in phospho-Stat1 fluorescence across the x-axis as represented by the orange histogram. However, when this same process is performed using the FoxP3 fix and perm buffer system, we now lose the ability to detect phospho-Stat1 (Fig. 7.36b).

The amount of time elapsed between harvesting stimulated cells and fixing them with paraformaldehyde is equally important as using the correct fix and perm combination. In Fig. 7.37, human monocytes were again stimulated with IFN γ , but this time, one group was fixed immediately with 1% Formaldehyde while a second group was first surface stained with a CD14 antibody for 30 min prior to fixation. Both groups were then permeabilized with methanol before being stained intracellularly with a phospho-specific Stat 1 antibody (Fig. 7.37). Cells that were immediately fixed after stimulation were stained for CD14 at the same time that staining for phospho-Stat 1 was performed. Despite all procedures being performed on ice, the

30 min spent staining for CD14 prior to fixation completely abolished the detection of phospho-Stat 1 (Fig. 7.37a, b) whereas phospho-Stat 1 can still be detected in cells fixed immediately after stimulation (Fig. 7.37c, d). The trade-off to staining after fixation is that not all antibodies designed to detect surface antigens are able to detect fixed surface antigens as well as they can detect unfixed surface antigens. Notice how the CD14 fluorescence is reduced when staining is performed after fixation (Fig. 7.37c, d) compared to when staining is performed before fixation (Fig. 7.37a, b).

Not all proteins will become dephosphorylated at the same rate and not all antibodies are compatible with fixed epitopes so it is crucial to determine the fixation/staining order for your own system. If surface staining must be performed before fixation and methanol permeabilization, special consideration needs to be made as to the fluorochromes used for your surface stains as not all fluorochromes are compatible with methanol treatment. This is especially true for large fluorochromes such as PE, PerCP, and APC. Methanol significantly reduces, or in some cases completely abolishes fluorescence by these fluorochromes. We cover this more extensively in Chap. 10, Sect. 10.2, Fig. 10.2. Additionally, a list of methanol-sensitive fluorochromes can be found in Table 9.1 (Chap. 9) and should be avoided on surface stains prior to 1% Formaldehyde fixation and permeabilization with Methanol. Colors such as PE, APC, and PerCP can still be used on intracellular phospho-specific antibodies AFTER methanol treatment but should not be used on surface antibodies that will be exposed to methanol treatment.

6 Materials

6.1 Flow Antibodies

- Mouse CD3 PE (FAB4841P; R&D Systems)
- Mouse CD45.1 BV421 (BD)
- Mouse CD45.2 BV605 (BD)
- Human CCR7 APC (FAB197A; R&D Systems)
- Human CCR7 PE (FAB197P; R&D Systems)
- Human CD3 (FAB100; R&D Systems)
- Human CD3 A750 (FAB100S; R&D Systems)
- Human CD3 A405 (FAB100V; R&D Systems)
- Human CD3 PE-Cy7*
- Human CD4 (FAB3791; R&D Systems)
- Human CD4 PE (FAB3791P; R&D Systems)
- Human CD4 A700 (FAB3791N; R&D Systems)
- Human CD4 A750 (FAB3791S; R&D Systems)
- Human CD4 A405 (FAB3791V; R&D Systems)
- Human CD8 PerCP (FAB1509C; R&D Systems)
- Human CD11b A700 (FAB16991N; R&D Systems)

- Human CD11c A700 (FAB1777N; R&D Systems)
- Human CD14 A405 (FAB3832V; R&D Systems)
- Human CD14 FITC (FAB3832F; R&D Systems)
- Human CD15 A488 (FAB2155G; R&D Systems)
- Human CD16 APC (FAB2546A; R&D Systems)
- Human CD16 FITC (FAB2546F; R&D Systems)
- Human CD19 A594 (FAB4867T; R&D Systems)
- Human CD19 A405 (FAB4867V; R&D Systems)
- Human CD25 (FAB1020; R&D Systems)
- Human CD25 APC (FAB1020A; R&D Systems)
- Human CD25 BV605 (BD)
- Human CD25 A700 (FAB1020N; R&D Systems)
- Human CD32 PE (FAB1330P; R&D Systems)
- Human CD45RA A488 (BioLegend)
- Human CD56 APC (FAB2408A; R&D Systems)
- Human CD62L A488 (FAB9787G; R&D Systems)
- Human CD63 PE (IC5048P; R&D Systems)
- Human CD64 FITC (FAB1906A; R&D Systems)
- Human CD127 APC (FAB306A; R&D Systems)
- Human CD304 PE (FAB3870P; R&D Systems)
- Human CXCR3 PE (FAB160P; R&D Systems)
- Human/mouse FoxP3 (IC8214; R&D Systems)
- Human $\gamma\delta$ TCR AmCyan (BD)
- Human HLA-DR PerCP (FAB4869C; R&D Systems)
- Human HLA-DR A594 (FAB4869T; R&D Systems)
- Human HLA-DR PerCP-Cy5.5 (BD)
- Human IFN- γ (IC285; R&D Systems)
- Human IFN- γ PE (IC285P; R&D Systems)
- Human Ki-67 (FAB7617; R&D Systems)
- Human LAMP1 A488 (IC4800G; R&D Systems)
- Human SLAMF7 APC (FAB1906A; R&D Systems)
- Human pSTAT1 (AF2894; R&D Systems)
- (*labeled with Novus lightning-link PE-Cy7 antibody labeling kit; 762–0010)

6.2 Isotype Control Antibodies

- Mouse IgG2a APC (IC003A; R&D Systems)
- Mouse IgG2b PE (IC0041P; R&D Systems)
- Rb IgG (IC1051; R&D Systems)
- Mouse IgG2b (MAB004; R&D Systems)

6.3 Proliferation and Viability Dyes and Reagents

- Annexin V BV421 (BD)
- 7-AAD (Life Technologies)
- CAM (NB5119; Novus Biologicals)
- Live-or-Dye™ 405/452 (Biotium)
- Live-or-Dye™ 405/545 (Biotium)
- ViaFluor® CFSE cell proliferation kit (Biotium)
- ViaFluor® 405 cell proliferation kit (Biotium)

6.4 Cell Activation Reagents

- MxhCD3 (MAB100; R&D Systems)
- GtxhCD28 (AF-342-PB; R&D Systems)
- Recombinant human IL-12 (219-IL; R&D Systems)
- Recombinant human IL-15 (247-ILB; R&D Systems)
- Recombinant human IL-18 (9124-IL; R&D Systems)
- Tocris cell activation cocktail (5476/1ML)
- Recombinant human IFN γ (285-IF; R&D Systems)
- Recombinant human IL-2 (202-IL; R&D Systems)
- Recombinant TGF- β (100-B; R&D Systems)
- PMA (Sigma)
- Ionomycin (Sigma)
- Monensin (Sigma)
- LPS (Sigma)

6.5 Cell Purification Kits

- MagCelect™ human CD3+ T cell isolation kit (MAGH101; R&D Systems)
- MagCelect™ human CD14+ cell isolation kit (MAGH105; R&D Systems)
- MagCelect™ human NK cell isolation kit (MAGH109; R&D Systems)

6.6 Staining Buffers/Fc Block Reagents

- FlowX FoxP3/transcription factor Fixation & Perm Buffer kit (FC012; R&D Systems)
- Normal human IgG (1-001-a; R&D Systems)
- Human Fc block:
 - hCD16 purified (AF1597; R&D Systems)

- hCD32 purified (AF1330; R&D Systems)
- hCD64 purified (AF1257; R&D Systems)
- Mouse IgG
- Flow cytometry staining buffer (FC001; R&D Systems)
- 1× fixation buffer (FC004; R&D Systems)
- 1× Saponin (FC005; R&D Systems)
- Flow Cytometry human lyse buffer (FC002; R&D Systems)

References

1. Pleass RJ, Woof JM. Fc-receptors and immunity to parasites. *Trends Parasitol.* 2001;17:545–51.
2. Gunn BM, Alter G. Modulating antibody functionality in infectious disease and vaccination. *Trends Mol Med.* 2016;22(11):969–82.
3. Goodlad RA. Quantification of epithelial cell proliferation, cell dynamics, and cell kinetics in vivo. *WIREs Dev Biol.* 2017;6:e274. <https://doi.org/10.1002/wdev.274>.
4. <https://biotium.com/products/cellular-assay-kits/cell-proliferation/>
5. Berhanu D, Mortari F, De Rosa SC, Roederer M. Optimized lymphocyte isolation methods for analysis of chemokine receptor expression. *J Immunol Methods.* 2003;279(1–2):199–207.
6. Paul S, Lal G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. *Front Immunol.* 2017;8:1–15.
7. Bryceson Y, et al. Functional analysis of human NK cells by flow cytometry. *Methods Mol Biol.* 2010;612:335–52.
8. Alter G, Malenfant J, Altfeld M. CD107a as a functional marker for the identification of natural killer cell activity. *J Immunol Methods.* 2004;294:15–22.
9. Gillissen MA, et al. The modified FACS calcein AM retention assay: a high throughput flow cytometer based method to measure cytotoxicity. *J Immunol Methods.* 2016;434:16–23.
10. Collin M, McGovern N, Haniffa M. Human dendritic cell subsets. *Immunology.* 2013;140(1):22–30.
11. Piccioli D, et al. Functional specialization of human circulating CD16 and CD1c myeloid dendritic-cell subsets. *Blood.* 2007;109(12):5371–9.
12. Matsuda Y, et al. Comparison of fixation methods for preservation of morphology, RNAs, and proteins from paraffin-embedded human cancer cell-implanted mouse models. *J Histochem Cytochem.* 2001;59(1):68–75.
13. Krutzik P, Nolan G. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. *Cytometry A.* 2003;55(2):61–70.
14. Jamur MC, Oliver C. Permeabilization of cell membranes. *Methods Mol Biol.* 2010;588:63.
15. Chew TS, et al. Optineurin deficiency in mice contributes to impaired cytokine secretion and neutrophil recruitment in bacteria-driven colitis. *Dis Model Mech.* 2015;8:17–29.
16. Mollenhauer HH, Morre DJ, Rowe LD. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim Biophys Acta.* 1990;1031(2):225–46.
17. Foster B, et al. Detection of intracellular cytokines by flow cytometry. *Curr Protoc Immunol.* 2007;78:6.
18. Balagopalan L, Barr V, Samelson L. Endocytic events in TCR signaling: focus on adapters in microclusters. *Immunol Rev.* 2009;232(1):84–98.
19. Albu D, et al. Flow cytometry analysis of transcription factors in T lymphocytes. *Transcription Factors.* 2010:377–90.

Chapter 8

Cell Enrichment



Christine Goetz, Christopher Hammerbeck, Andrew Wyman,
and Jae-Bong Huh

1 Enrichment of Cell Populations Using Columns

The general principle behind column enrichment is to load antibody-labeled mononuclear cell suspensions onto enrichment columns containing glass beads coated with immunoglobulins (Ig) and anti-Ig. For column enrichment, the selection process used is high affinity negative selection. For example, general T cell subsets (CD3⁺, CD4⁺, or CD8⁺) or naive cell subsets (e.g., CD4⁺CD45RO⁻) can be purified in mouse, rat, or human cells. Mini, small, and large columns are available to support the number of cells that need to be purified. To start the process of column enrichment, columns are first washed with 1× column buffer to equilibrate the columns, and then leukocytes are incubated with the desired monoclonal antibody cocktails. Cells are then washed with 1× column buffer and loaded onto the equilibrated column. After a short incubation in the column, the cells are washed multiple times with 1× column wash buffer, and the eluate containing the desired cells are washed and then used in downstream applications (Table 8.1).

For example, with CD4⁺ T cell selection, leukocyte suspensions are incubated with a mixture of monoclonal antibodies and then loaded onto T Cell Subset Columns (see Fig. 8.1). B cells and nonselected T cell subsets bind to anti-Ig-coated glass beads via F(ab)-surface Ig interactions, while monocytes bind to Ig-coated glass beads via Fc interactions. The resulting column eluate contains a highly enriched T cell subset population with virtually no B cells, monocytes, or nonselected T cell subsets. The purity of the desired cells populations is usually between 85 and 90%.

C. Goetz (✉) · C. Hammerbeck · A. Wyman · J.-B. Huh
Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA
e-mail: Christine.Goetz@bio-techno.com; Chris.Hammerbeck@bio-techno.com; Andrew.Wyman@bio-techno.com; Jae-Bong.Huh@bio-techno.com

Table 8.1 Pros and cons of sorting versus column/magnetic purification

Method of cell enrichment	Pros	Cons
Column-based purification/ magnetic purification	– Negative selection allows for enrichment of cells with no antibody attached to them – Cells are not stressed with high pressure sorting – No limitations on the number of cells that can be purified – Less expensive than sorting (magnets are a one-time investment)	– Positive selection has an antibody/antibodies attached to your enriched cell population which may interfere with downstream applications – Scaling antibodies to smaller cell preps is not always linear and requires optimization – Cell purity is rarely over 90%
Sorting	– No limit on the number of cells that can be purified – Up to 4 populations can be sorted simultaneously so it is time efficient – Single cell sorts into 96-well plates are possible – Cell purity is usually >95%	– Sorting can stress your cells (high pressure is required for sorting) and may affect downstream ex vivo assays – Sorting is expensive (usually ~ \$200+/h)

2 Magnetic Selection Principles and Types of Selection

Magnetic cell selection is a simple, yet effective method to enrich a specific cell type within a mixed population of cells such as PBMCs or cells from secondary lymphoid organs such as splenocytes. Magnetic selection is based on the use of ferrofluids or magnetic nanoparticles (“beads”) that have no magnetic memory (superparamagnetic) and behave like colloidal particles. These magnetic beads are typically conjugated to streptavidin, which then binds biotin-conjugated antibodies.

Cells are first incubated with a biotin-conjugated antibody or biotinylated antibody cocktail. Streptavidin ferrofluid is next added and the streptavidin-coated beads interact with the biotinylated antibody-tagged cells, forming an antibody-bead complex. When the sample is placed within a magnetic field, the tagged cells (those with the biotin-streptavidin bead complex bound) migrate toward the magnet leaving the untagged cells in suspension. The enriched cell preparation is then available for a variety of applications including tissue culture, immune status monitoring, and flow cytometry. There are two distinct types of magnetic selection, positive selection and negative selection (refer to Fig. 8.2).

Negative selection is when the undesired cell population(s) is tagged and magnetically separated, leaving the desired population untouched (see Fig. 8.2a). Positive selection is when the desired cell population is tagged and magnetically separated (see Fig. 8.2b). Depending on the post-enrichment application, untagged cells may be preferred. There are several ready-made kits available on the market for magnetic cell selection. They typically consist of a biotinylated antibody, streptavidin-coated ferrofluid, a cell buffer, and a specialized magnet, all optimized

T Cell Enrichment Columns Assay Principle

For purification of T cells by high affinity negative selection.

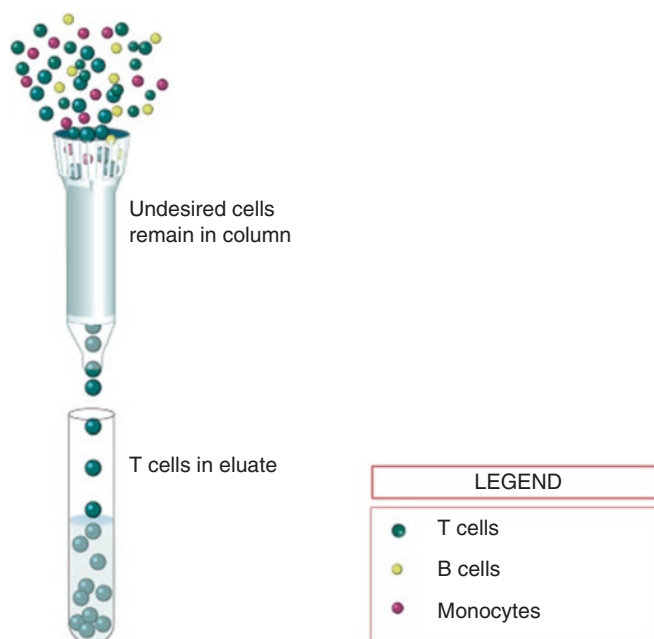


Fig. 8.1 Enrichment of T cells using columns. The schematic illustrates the CD4 T cell enrichment column assay principle using high affinity negative selection. PBMCs or rodent splenocytes are labeled with an antibody cocktail, and then placed over an equilibrated column. T cells (aqua colored cells) pass through the column, and the undesired cells (such as B cells shown in green and monocytes shown in red) remain. T cells are collected in the eluate, and can be used in downstream applications

for use together. When using standalone products, you will have to optimize the amount of reagents to use on your own, which means finding the correct antibody-to-bead ratio in relation to the starting population size. Figure 8.3a shows how magnetic negative selection can be used to enrich for two cell populations: $hCD4^+CD45RO^+$ Memory T cells. The pre- and post-purification data is shown in Fig. 8.3b, c. CCR7 serves as a marker for Memory T cells along with CD45RO. $CD4^+CD45RO^+$ and $CCR7^+CD45RO^+$ T cells are highly enriched using this magnetic purification kit (from 39% and 35% to 86% and 87%, respectively). As with the column method, the purity of the desired cells populations is usually between 85–90%.

Using too many cells (or too little antibody and/or beads) may affect both your target cell purity and recovery. To make things more complicated, the amount of biotinylated antibody and ferrofluid is not always linear. For example, starting with double the amount of cells isn't always as simple as doubling the amount of bioti-

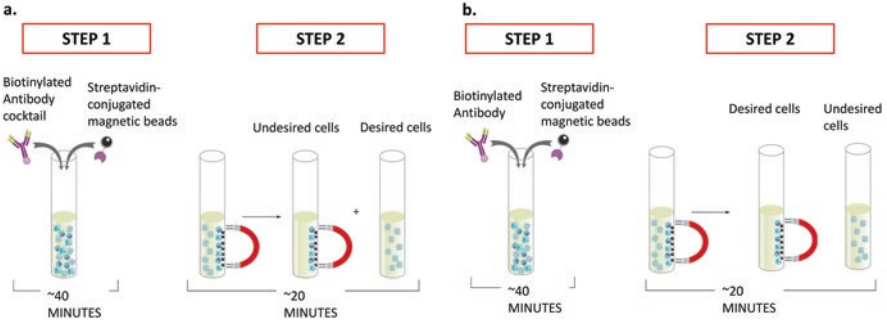


Fig. 8.2 Schematic of negative and positive Selection using magnetic beads. Negative or positive selection using magnetic beads is a two-step process. **(a)** The desired cells are incubated with a biotinylated antibody cocktail, streptavidin ferrofluid is added, and then the labeled cells are placed on a magnet. The desired cells are present in the supernatant, and undesired cell fraction is bound to the magnet. **(b)** The desired cells are incubated with a biotinylated antibody cocktail, streptavidin ferrofluid is added, and then the labeled cells are placed on a magnet. The undesired cells are present in the supernatant, and desired cell fraction is bound to the magnet. Note that cells purified using positive selection will have magnetic beads attached. In either case, the purified cell population can be used in downstream applications

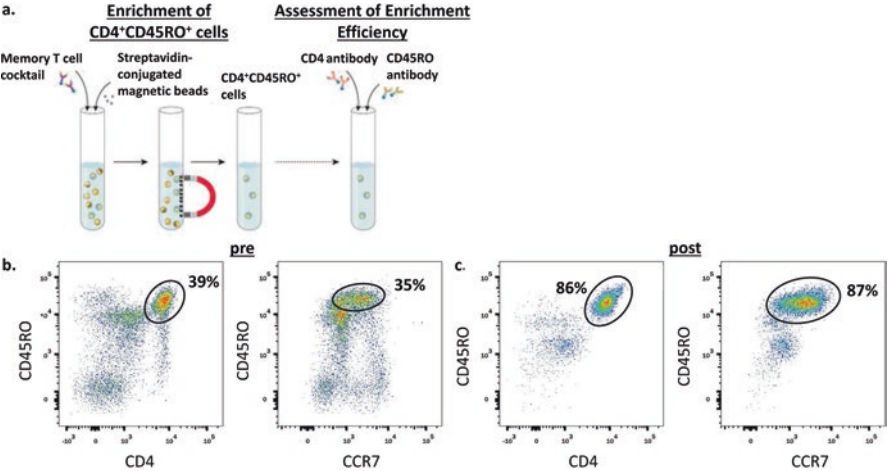


Fig. 8.3 Schematic of hCD4⁺ Memory T cell purification. Negative selection is used to optimally enrich hCD4⁺CD45RO⁺ T cells. **(a)** A biotinylated depletion cocktail is used to purify the hCD4⁺CD45RO⁺ T cells (left panel). The final step involves assessing cell purity using antibodies against CD4 and CD45RO (right panel). Purity is usually ~80–90% using this method. **(b, c)** Pre- and post-purification data. PBMCs **(b)** and purified CD4⁺CD45RO⁺ T cells **(c)** were stained with CD4, CD45RO, and CCR7 to assess pre- and post-purification cell purity

nylated antibody and streptavidin ferrofluid. Without proper optimization, your enrichment purity and cell recovery can be greatly affected. Generally speaking, there are guidelines for scaling up or scaling down magnetic purifications, so these can serve as a starting point. Researchers can then carry out titrations for the biotinylated antibody (or cocktails) and streptavidin beads to optimize these amounts for their particular application.

3 Fluorescence Activated Cell Sorting (FACS)

Fluorescence Activated Cell Sorting (FACS) is the process known for separation of live cell populations into further subpopulations based upon the fluorochrome that is associated with a detection antibody. FACS is a derivative of flow cytometry, where the end user can physically sort a heterogeneous population into 1–4 distinct cell populations. When cell sorting is carried out, cells are prepared in the same manner as with standard flow cytometry where cells are labeled with antibodies conjugated to fluorochromes. Separation of these cells is performed on flow cytometers specifically designed for this purpose.

In most cases, cells are diverted into a single stream that passes through a nozzle, which can vary in diameter based upon the cells of interest. These nozzles create a single droplet, which in theory represents a single cell and carries a specific charge, which can then be processed at an interrogation point. This interrogation point then allows the machine to determine how the droplet should be separated based upon charge. This separation is performed as the droplet passes through a set of deflection plates, which can influence the direction of the droplet based upon charge (either positive or negative). Depending on how you set up your experiment, 1–4 cell populations can be sorted simultaneously.

Figure 8.4a shows an image of a sort being carried out where two distinct cell populations are being sorted. Shown below in Fig. 8.4b is an example of a 2-way sort where T-cells (CD3⁺) and B-cells (B220⁺) have been sorted in mouse splenocytes. The gates for B220 PE and CD3 APC are set based on isotype controls (Rat IgG2a PE and Rat IgG2b APC, respectively). The pre-sort values for CD3⁺ and B220⁺ cells are approximately 32.9% and 62.8%, respectively. Post-sort values show highly enriched populations of both B and T cells, with purities of >99% in both cases. Purities are typically higher in sorted cell populations versus those that are enriched using columns or magnets. With that in mind, sorted cells are ideal for carrying out qRT-PCR as the cell populations need to be very pure to accurately assess gene expression using this method. However, cell enrichment using the magnets and columns is sufficient for polarization of T cells and other cell lineages. The enrichment process ensures that the majority of cells that will expand in culture are of the desired lineage, and along with the optimal cytokine cocktails, polarization to a specific cell subset is achievable.

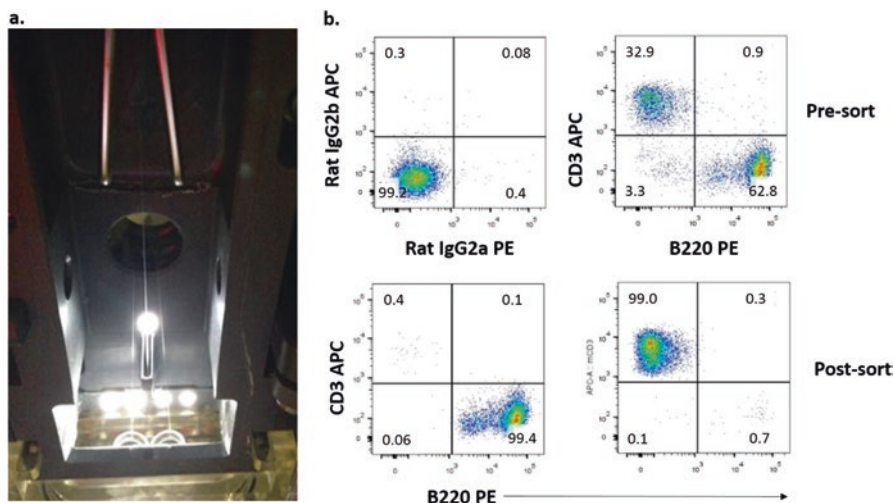


Fig. 8.4 Sorting on the BD FACSaria™. Sorting cells by flow cytometry involves the same preparation as acquisition. Mouse splenocytes were stained with B220 PE and CD3 APC (or isotype controls), and the sort gates were set on the desired cell populations. **(a)** This is an image of a 2-way sort being carried out on the Aria using mouse splenocytes labeled with CD3 and B220. The streams carrying highly purified CD3⁺ T cells or B cells are diverted into separate collection tubes. **(b)** Here are the corresponding dot plots showing the pre- and post-sort data. The top panels show the pre-sort gates for CD3 and B220 (right dot plot) set based off the isotype controls (left dot plot). The bottom panels show the post-sort data. The left plot shows highly purified CD3⁺ T cells, and the right dot plot shows highly purified B cells using B220. Each sorted cell population is around >99% pure

4 Materials

4.1 Flow Antibodies

- Mouse B220 PE (FAB1217P; R&D Systems)
- Mouse CD3 APC (FAB4841A; R&D Systems)
- Human CCR7 (FAB197; R&D Systems)
- Human CD4 (FAB3791; R&D Systems)
- Human CD45RO (K0015-4; R&D Systems)

4.2 Isotype Controls

- Rat IgG2b APC (IC013A; R&D Systems)
- Rat IgG2a PE (IC006P; R&D Systems)

4.3 Cell Purification Kits

- Mouse CD4⁺ T cell enrichment column, small (MCD4C-1000; R&D Systems)
- Mouse CD4⁺ T cell enrichment column, mini (MCD43; R&D Systems)
- Human CD4⁺ T cell enrichment column, small (HCD4C-1000; R&D Systems)
- Human CD4⁺ T cell enrichment column, mini (HCD43; R&D Systems)
- Rat CD4⁺ T cell enrichment column, small (RCD4C-1000; R&D Systems)
- MagCelect™ human memory CD4⁺ T cell isolation kit (MAGH116; R&D Systems)

4.4 Staining Buffers/Fc Block Reagents

- MagCelect plus 10× buffer (895921; R&D Systems)
- Normal human IgG (1-001-A; R&D Systems)
- Normal mouse IgG
- Flow Cytometry staining buffer (FC001; R&D Systems)
- Flow Cytometry human lyse buffer (FC002; R&D Systems)

Chapter 9

Surface and Intracellular Staining Protocols for Flow Cytometry



Christine Goetz and Christopher Hammerbeck

1 Staining Buffers for Flow Cytometry

In general, flow cytometry staining buffer (also known as FACS Buffer) is a PBS (Phosphate Buffered Saline)-, or HBSS (Hanks Balanced Salt Solution)-based solution that contains a source of protein such as Bovine Serum Albumin (BSA), Fetal Bovine Serum (FBS), or Fetal Calf Serum (FCS). Either PBS or HBSS can be used for flow staining buffer. Both are isotonic salt solutions that help maintain a neutral pH and osmotic pressure, but PBS is a buffered salt solution while HBSS is a balanced salt solution. HBSS contains glucose, which can help maintain cell viability during longer procedures, whereas PBS has a larger capacity to maintain pH. Cell culture media is typically not recommended as a flow cytometry staining media due to the CO₂-carbonate buffering system used in most cell culture media. Cell culture media are designed to be used at higher CO₂ concentrations such as those found in cell culture incubators which are around 5%. At lower CO₂ concentrations such as 0.04%, which is the typical atmospheric concentration, the CO₂ in cell culture media evaporates leading to a rise in pH. The increased alkalinity of the cell culture media can then lead to a decrease in cell viability which can negatively affect flow cytometry results.

The presence of proteins such as BSA, FBS, or FCS helps minimize nonspecific heterophilic binding of antibodies and helps maintain the viability of cells. The concentration of protein can vary but the most common concentration for BSA is between 0.5 and 1% whereas the most common concentrations for FBS and FCS are between 2 and 5%. Rarely does the concentration of protein exceed 5% as this can negatively affect antibody binding and can contribute to autofluorescence. Higher protein concentrations can also negatively affect cell sorting by causing variations

C. Goetz (✉) · C. Hammerbeck

Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA
e-mail: Christine.Goetz@bio-technie.com; Chris.Hammerbeck@bio-technie.com

© Springer Nature Switzerland AG 2018

C. Goetz et al., *Flow Cytometry Basics for the Non-Expert*, Techniques in Life Science and Biomedicine for the Non-Expert,
https://doi.org/10.1007/978-3-319-98071-3_9

157

in flow rates, so for flow sorting experiments, sorting media should ideally contain less than 2% FBS/FCS or 0.2%–1% BSA.

Cell aggregation can negatively affect flow cytometry and flow sorting results and can result in doublet events. This can be the result of more adherent cells, such as macrophages or adherent cell lines, or can be the result of the release of DNA during cell death. To alleviate the possibility of cell aggregation, EDTA (typically around 0.5–5 mM) can be added to staining and sorting buffers to reduce the number of doublets by limiting cell-cell interactions. EDTA reduces calcium-dependent cell adhesion molecule and integrin interactions by sequestering Ca^{2+} and Mg^{2+} ions, thus preventing cells from interacting during staining procedures. The addition of DNase (25–200 $\mu\text{g}/\text{mL}$) can prevent aggregation caused by free DNA, especially when using fragile cells such as neutrophils, which have a short lifespan and undergo apoptosis in response to even gentle manipulations. One important consideration when using both EDTA and DNase in staining or sorting buffers is that DNase requires the use of Mg^{2+} ions to be effective. EDTA will chelate Mg^{2+} ions, limiting the effectiveness of DNase treatments. If DNase and EDTA must be used together, EGTA should be used in place of EDTA. EGTA will still sequester Ca^{2+} ions, preventing cell adhesion molecule and integrin interactions, but has a lower effect on Mg^{2+} ions. Using Ca^{2+} - and Mg^{2+} -free PBS or HBSS can also help limit cell-cell interactions but will negatively affect the efficacy of DNase treatment.

For long-term storage of FACS buffer, a small amount of sodium azide (usually 0.05–1%) is added. Sodium azide is bacteriostatic and inhibits the growth of certain bacteria in bulk reagents and stock solutions. If FACS buffer is made fresh and intended for a single, same-day use, the inclusion of sodium azide is not necessary. Aside from preventing the outgrowth of bacteria in flow cytometry staining buffer, the presence of sodium azide can improve the detection of cell proteins that are continuously recycled from the cell surface to intracellular compartments, such as chemokine receptors, as sodium azide is an endocytosis inhibitor.

1.1 Key Tips for Surface and Intracellular Staining

- Incubations with directly conjugated antibodies must be done in the dark; exposure of your cells to light for prolonged periods of time can photobleach your cells. This is especially important to keep in mind with direct conjugates using tandem dyes (e.g., PE-Cy7). Tandem dyes are less stable, and exposure to light can cause them to fall apart.
- Staining temperatures have been optimized for each of these protocols; be sure to follow them.
- Do not skip the Fc receptor blocking steps. This is critical, especially if you are staining monocytes that have a lot of Fc receptors. Your stains will be messy and difficult to interpret if you do not use Fc block.

- For human IgG, mouse IgG, or rat IgG, use 5 µg/100 µl cells.
- If using anti-CD16/32, use 1 µg/100 µl cells.
- Be sure to use a centrifuge that goes up to at least 1250 rpm. Spinning cells at too low of a speed will cause you to lose a lot of your cells. This is especially critical when carrying out intracellular staining as the cell pellets become loose with the permeabilization buffers. For laboratories using single-speed serological centrifuges, 1 min at between 3250 and 3500 rpm ($1049\text{--}1,999 \times g$) will be sufficient for unpermeabilized cells; 2 min for fixed and permeabilized cells. When using larger variable-speed tabletop centrifuges, 5 min at 1250–1500 rpm ($350\text{--}500 \times g$) will be sufficient for unpermeabilized cells; 10 min at 1500–2000 rpm ($500\text{--}950 \times g$) would be recommended for fixed and permeabilized cells.
- Be sure to dilute buffers as recommended. Over-fixing your cells can dim the fluorochromes (see Chap. 10). In addition, using too high of a concentration of permeabilization buffer may result in destroying the cell membrane and ruin your experiment for the detection of your intracellular antibody.
- Take care to avoid using PE or APC conjugates prior to methanol permeabilization (see Table 9.1 below for recommendations). Methanol destroys the PE and APC epitopes causing you to either lose your stain with a low-density marker or dim it with a high-density marker (see Chap. 10 for more information).

2 Surface Staining: Tips and Tricks

There are various protocols available for cell surface staining, and in this section, we will go over a few of them. We will list protocols for staining for unconjugated antibodies and direct conjugates in whole blood and PBMCs, biotinylated antibodies, chemokine receptor staining, and detection of LAMP proteins. The methodology varies slightly for each one. These protocols can also be applied to staining whole blood and splenocytes from rodents (mice and rats). The main difference with staining rodent cell populations is that the lyse buffers are slightly different

Table 9.1 Fluorochromes sensitive and resistant to Methanol

Methanol sensitive fluorochromes	Methanol resistant fluorochromes
PE	A488
PE-tandems (e.g., PE-Cy7, etc.)	A647
PerCP	A405
PerCP-tandems (e.g., PerCP-Cy5.5)	A594
APC	A700
APC-tandems (e.g., APC-Cy7)	A750
	FITC

Abbreviations used: *IC* Intracellular, *GAM* Goat anti-mouse, *WB* Whole blood, *Msxh* Mouse anti-human

(e.g., use mouse lyse buffer for mouse and rat tissues) and blocking reagents must be matched to the species you are staining for. Keep in mind that this is a starting point, and these protocols can be optimized by the end user for their particular set of antibodies.

2.1 Cell Surface Staining in Whole Blood (WB)

2.1.1 Cell Surface Staining for Unconjugated Antibodies in Human WB

In order to apply this protocol to mouse or rat WB, use 1× Mouse Lyse. For blocking mouse WB, use mouse IgG. For rat WB, use rat IgG to block. Blocking reagents that are applied between the secondary and costain need to be the same as the host of the costain antibodies you are using (e.g., for MxhCD3, the host is mouse so Mouse IgG block is needed).

Materials needed:

1× human lyse buffer

Human IgG block, mouse IgG block, or Fc block

Flow cytometry staining buffer

12×75 mm polypropylene flow tubes (hereafter referred to as flow tubes)

Unconjugated antibody

Secondary antibody conjugated to a fluorochrome (e.g., Goat anti-mouse PE)

Directly conjugated costains (if needed)

Note: Carry out staining at room temperature

1 mL of unwashed WB will yield enough blood for approximately seven tubes

1. Wash ~1 mL of WB with 2 mL Flow cytometry staining buffer, three times. This removes the plasma. Blood pellets are looser and can't be dumped out. Remove the plasma with a transfer pipette. For washes, spin cells in centrifuge for 1 min (3100–3500 rpm/1049–1999 × *g*) to 5 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.
2. Blood needs to be blocked with human IgG. Monocytes have Fc receptors, which bind antibodies nonspecifically. If you do not block, everything will look positive. Human IgG will block those Fc receptors so that the antibody attaches to its intended, specific receptor. Block with human IgG for 10 min at room temperature, and do not wash it out.
3. Pipette ~75 µL of packed WB into each tube. If you are targeting a smaller population of cells (such as monocytes or NK cells) you may want to add more blood (~100 µL) to each tube.
4. Add the primary antibody to each tube and incubate for 30 min at room temperature.
5. Wash twice with 2 mL Flow cytometry staining buffer. Remember not to dump out the tube after spinning. You must use a transfer pipette to remove the liquid.

6. Add the secondary antibody to each tube and incubate for 20 min at room temperature in the dark.
7. If a costain is required, wash cells twice with 2 mL flow cytometry staining buffer. You must use a transfer pipette to remove the liquid. Then Fc block with the host of the species of antibody you are using. For example, if your costains are Mxsh, then use mouse IgG block. Add mouse IgG and let it incubate for 10 min at room temperature; do not wash it out. Then add the directly conjugated antibody/antibodies for 20 min. If no costains are required, proceed to **step 8**.
8. Add 2–3 mL of 1× human lyse buffer to each tube, and vortex each tube thoroughly to mix. This will lyse all of the red blood cells (RBCs) and leave only the white blood cells. Incubate for 10 min at room temperature in the dark, vortex each tube again, and incubate for an additional 10 min. The blood should turn from opaque to clear if the lysis is good.
9. Spin the cells down and wash several times with flow cytometry staining buffer (usually 2–3 times) until most of the RBCs have been removed.
10. Resuspend cells in ~400 µL flow cytometry staining buffer for analysis and run on a flow cytometer.

2.1.2 Cell Surface Staining for Directly Conjugated Antibodies in Human WB

In order to apply this protocol to mouse or rat WB, use 1× mouse lyse buffer. For blocking mouse WB, use mouse IgG. For rat WB, use rat IgG to block.

Materials needed:

1× human lyse buffer

Human IgG block, mouse IgG block, or Fc block

Flow cytometry staining buffer

Flow tubes

Directly conjugated antibody/antibodies

Note: Carry out staining at room temperature

1 mL of unwashed WB will yield enough blood for approximately seven tubes

1. Wash ~1 mL of WB with 2 mL Flow cytometry staining buffer, three times. This removes the plasma. Blood pellets are looser and can't be dumped out. Remove the plasma with a transfer pipette. For washes, spin cells in centrifuge for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
2. Blood needs to be blocked with human IgG. Monocytes have Fc receptors, which bind antibodies nonspecifically. If you don't block, everything will look positive. Human and mouse IgG will block those Fc receptors so that the antibody attaches to its intended, specific receptor. Block with human IgG for 10 min at room temperature, and do not wash it out.

3. Pipette $\sim 75 \mu\text{L}$ of packed WB into each tube. If you are targeting a smaller population of cells (such as monocytes or NK cells) you may want to add more blood ($\sim 100 \mu\text{L}$) to each tube.
4. Add the directly conjugated antibody or antibodies to each tube and incubate for 30 min at room temperature. (Direct conjugates can be stained at the same time; there is no need to have separate incubations for each one.)
5. Add 2–3 mL of $1\times$ human lyse buffer to each tube, and vortex each tube thoroughly to mix. This will lyse all of the red blood cells (RBCs) and leave only the white blood cells. Incubate for 10 min at room temperature in the dark, vortex each tube again, and incubate for an additional 10 min. The blood should turn from opaque to clear if the lysis is good.
6. Spin the cells down and wash several times with Flow cytometry staining buffer (usually 2–3 times) until most of the RBCs have been removed.
7. Resuspend cells in $\sim 400 \mu\text{L}$ flow cytometry staining buffer for analysis and run on a flow cytometer.

2.2 Cell Surface Staining in Peripheral Blood Mononuclear Cells (PBMCs)

2.2.1 Cell Surface Staining for Unconjugated Antibodies in PBMCs

This staining protocol can also be applied to cells that have been differentiated or stimulated with various reagents to induce upregulation of surface markers (e.g., Th1 differentiation, PMA/Ionomycin, etc.). In addition, mouse or rat secondary lymphoid organs (spleen, lymph nodes, etc.) can be stained using these protocols. As stated above, for mouse tissues, block with mouse IgG; for rat tissues, block with rat IgG. Blocking reagents that are applied between the secondary and costain need to be the same as the host of the costain antibodies you are using (e.g., For MsxhCD3, the host is mouse so the block needed is mouse IgG).

Materials needed:

Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer
Flow tubes
Unconjugated antibody
Secondary antibody conjugated to a fluorochrome (e.g., Goat anti-mouse PE)
Directly conjugated costains (if needed)

Note: Carry out staining at room temperature

1. Wash cells twice to remove media: Add 2 mL Flow cytometry staining buffer, vortex, and spin for 1 min ($3100\text{--}3500 \text{ rpm}/1049\text{--}1999 \times g$) to 5 min ($1250\text{--}1500 \text{ rpm}/350\text{--}500 \times g$) depending on the centrifuge used.
2. Resuspend cells in enough flow cytometry staining buffer for your experiment (e.g., if you have 8 tubes, resuspend the cells in at least $800 \mu\text{L}$ flow cytometry

staining buffer so you will end up with 100 μL per tube). Block Fc receptors using human IgG for 10 min at room temperature. During this 10 min incubation, aliquot 100 μL cells into each tube. There should be between 100,000 and 1,000,000 cells in each tube.

3. Add the primary antibody to the cells. Vortex and incubate for 30 min at room temperature.
4. Wash cells twice in 2 mL flow cytometry staining buffer as above.
5. Add the secondary antibody and incubate for 20 min at room temperature in the dark.
6. Wash cells twice in 2 mL flow cytometry staining buffer as above.
7. If a costain is required, block prior to adding the costain antibody/antibodies. Fc block with the host of the species of antibody you are using. For example, if your costains are Mxsh, then use mouse IgG block. To do this, add mouse IgG and let it incubate for 10 min at room temperature; do not wash it out. Add the directly conjugated antibody/antibodies for 20 min. Then wash cells twice in 2 mL flow cytometry staining buffer. If no costains are required, proceed to **step 8**.
8. Resuspend cells in ~400 μL flow cytometry staining buffer for flow cytometry analysis.

2.2.2 Cell Surface Staining for Directly Conjugated Antibodies in PBMCs

This staining protocol can also be applied to cells that have been differentiated or stimulated with various reagents to induce upregulation of surface markers (e.g., Th1 differentiation, PMA/Ionomycin, etc.). In addition, mouse or rat secondary lymphoid organs (spleen, lymph nodes, etc.) can be stained using these protocols. As stated above, for mouse tissues, block with mouse IgG; for rat tissues, block with rat IgG.

Materials needed:

Human IgG block, mouse IgG block, or Fc block

Flow cytometry staining buffer

Flow tubes

Directly conjugated antibody/antibodies

Note: Carry out staining at room temperature

1. Wash cells twice to remove media: Add 2 mL flow cytometry staining buffer, vortex, and spin for 1 min (3100–3500 rpm/1049–1999 $\times g$) to 5 min (1250–1500 rpm/350–500 $\times g$) depending on the centrifuge used.
2. Resuspend cells in enough flow cytometry staining buffer for your experiment (100 μL per tube). Block Fc receptors using human IgG and mouse IgG for 10 min at room temperature. During this 10 min incubation, aliquot 100 μL cells into each tube (e.g., if you have 8 tubes, resuspend the cells in at least 800 μL flow cytometry staining buffer). There should be between 100,000 and 1,000,000 cells in each tube.

3. Add each antibody/antibodies to the cells. Vortex and incubate for 30 minutes at room temperature.
4. Wash cells twice in 2 mL Flow cytometry staining buffer as above.
5. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

2.3 Cell Surface Staining for Biotinylated Antibodies

This staining protocol is virtually identical to surface staining for unconjugated antibodies in WB and PBMCs. The biotin-streptavidin interaction is one of the strongest interactions in nature and is well characterized. Streptavidin exists as a tetramer where each subunit binds 1 biotin molecule. The interaction is highly specific, and is not as sensitive to denaturing reagents and buffers as other primary antibody/secondary antibody complexes are (Expedeon; The Biotin Streptavidin Interaction; 2017). The biotinylated antibody is the primary antibody, and fluorochrome-conjugated Streptavidin is the secondary reagent that is required for detection. The major difference is that the Streptavidin secondary reagent is not species specific; therefore, you will not have issues with messy staining between secondary antibodies and costains. With that in mind, you can eliminate the block step between secondary antibodies and costains.

2.3.1 Cell Surface Staining for Biotinylated Antibodies in WB

In order to apply this protocol to mouse or rat WB, use 1 $\times$ Mouse Lyse. For blocking mouse WB, use mouse IgG. For rat WB, use rat IgG to block.

Materials needed:

1 $\times$ human lyse buffer

Human IgG block, mouse IgG block, or Fc block

Flow cytometry staining buffer

Flow tubes

Biotinylated antibody

Streptavidin conjugated to a fluorochrome (e.g., Streptavidin PE)

Directly conjugated costains (if needed)

Note: Carry out staining at room temperature

1 mL of unwashed WB will yield enough blood for approximately seven tubes

1. Wash ~1 mL of WB with 2 mL flow cytometry staining buffer, three times. This removes the plasma. Blood pellets are looser and can't be dumped out. Remove the plasma with a transfer pipette. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.

2. Blood needs to be blocked with human IgG and mouse IgG. Monocytes have Fc receptors, which bind antibodies nonspecifically. If you don't block, everything will look positive. Human IgG and mouse IgG will block those Fc receptors so that the antibody attaches to its intended, specific receptor. To do this, add both human IgG and mouse IgG for 10 min at room temperature; do not wash it out.
3. Pipette ~75 μ L of packed WB into each tube. If you are targeting a smaller population of cells (such as monocytes or NK cells) you may want to add more blood (~100 μ L) to each tube.
4. Add the biotinylated antibody to each tube and incubate for 30 min at room temperature.
5. Wash two times with 2 mL flow cytometry staining buffer. Remember not to dump out the tube after spinning. You must use a transfer pipette to remove the liquid.
6. Add Streptavidin PE (or other fluorochromes) to each tube and incubate for 20 min at room temperature in the dark.
7. If a costain is required, wash cells two times with 2 mL flow cytometry staining buffer. You must use a transfer pipette to remove the liquid. Then add the directly conjugated antibody/antibodies for 20 min. If no costains are required, proceed to **step 8**.
8. Add 2–3 mL of 1 $\times$ human lyse buffer to each tube, and vortex each tube thoroughly to mix. This will lyse all of the red blood cells and leave only the white blood cells. Incubate for 10 min at room temperature in the dark, vortex each tube again, and incubate for an additional 10 min. The blood should turn from opaque to clear if the lysis is good.
9. Spin the cells down and wash several times with flow cytometry staining buffer (usually two to three times) until most of the RBCs have been removed.
10. Resuspend cells in ~400 μ L Flow cytometry staining buffer for analysis and run on a flow cytometer.

2.3.2 Cell Surface Staining for Biotinylated Antibodies in PBMCs

This staining protocol can also be applied to cells that have been differentiated or stimulated with various reagents to induce upregulation of surface markers (e.g., Th1 differentiation, PMA/Ionomycin, etc.). In addition, mouse or rat secondary lymphoid organs (spleen, lymph nodes, etc.) can be stained using these protocols. As stated above, for mouse tissues, block with mouse IgG; for rat tissues, block with rat IgG.

Materials needed:

Human IgG block, mouse IgG block, or Fc block

Flow cytometry staining buffer

Flow tubes

Biotinylated antibody

Streptavidin conjugated to a fluorochrome (e.g., Streptavidin PE)

Directly conjugated costains (if needed)

Note: Carry out staining at room temperature

1. Wash cells two times to remove media: Add 2 mL flow cytometry staining buffer, vortex, and spin for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.
2. Resuspend cells in enough flow cytometry staining buffer for your experiment (e.g., if you have 8 tubes, resuspend the cells in at least 800 μ L flow cytometry staining buffer so you will end up with 100 μ L per tube). Block Fc receptors using human IgG and mouse IgG for 10 min at room temperature. During this 10 min incubation, aliquot 100 μ L cells into each tube. There should be between 100,000 and 1,000,000 cells in each tube.
3. Add the biotinylated antibody to the cells. Vortex and incubate for 30 min at room temperature.
4. Wash cells two times in 2 mL flow cytometry staining buffer as above.
5. Add Streptavidin PE (or other fluorochrome) and incubate for 20 mins at room temperature in the dark.
6. Wash cells two times in 2 mL flow cytometry staining buffer as above.
7. If a costain is required, add the directly conjugated antibody/antibodies for 20 min. Then wash cells two times in 2 mL flow cytometry staining buffer. If no costains are required, proceed to **step 8**.
8. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

2.4 Cell Surface Staining for Chemokine Receptors

Chemokine receptors undergo internalization and endocytic recycling which can be induced by even subtle manipulations such as temperature shifts, media changes during Ficoll separation, and lysis of whole blood. As a consequence, receptor internalization may render the cell negative. The addition of sodium azide prior to manipulation of cells may prevent internalization of membrane receptors; however, staining for chemokine receptors at higher temperatures, such as 37 °C, most often allows for maximal detection because higher temperatures: (1) allow for both the receptor and cell membrane to remain fluid, increasing the likelihood that the receptors will enter the correct conformation to be recognized by receptor-specific antibodies, and (2) allow chemokine receptors that have been internalized to be recycled to the cell surface exposing the greatest number of receptors to antibodies. Not all chemokine receptors require higher temperatures for detection but in the absence of a positive chemokine receptor staining, increasing the staining temperature in a good first step to consider when troubleshooting. Note, this protocol is not limited to chemokine receptors but can also be used on cytokine receptors or other surface proteins for which expression is difficult to detect by conventional flow cytometry staining protocols at 4 °C.

Materials needed:

Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer with sodium azide
Flow tubes

Directly conjugated antibody/antibodies

1. Obtain desired cells and prepare a single-cell suspension in flow cytometry staining buffer.
2. Wash cells two times in flow cytometry buffer. Add 2–3 mL flow cytometry buffer, vortex, and centrifuge for 1 min (3100–3500 rpm/1049–1999 $\times g$) to 5 min (1250–1500 rpm/350–500 $\times g$) depending on the centrifuge used.
3. Resuspend cells in flow cytometry buffer to a concentration of 5×10^6 – 1×10^6 cells/100 μ L and add 100 μ L of cells into 12 $\times$ 75 mm flow tubes.
4. Block for Fc receptors using a combination of human and mouse IgG. Incubate cells with blocking IgG for 10 min at room temperature.
5. Add the appropriate chemokine receptor antibody or isotype control antibody, vortex gently to mix, and incubate for 15–20 min in a 37 °C incubator.
6. Remove cells from the incubator and add any necessary primary antibodies/isotype control antibodies to detect other cell surface proteins. Vortex gently to mix and incubate for 20–30 min at room temperature.
7. Wash cells two times in 2–3 mL flow cytometry staining buffer as above.
8. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

2.5 Cell Surface Staining for LAMP Proteins

LAMP glycoproteins are expressed on the luminal surface of cytolytic vesicles during NK cell degranulation. There are 3 family members: LAMP-1 (CD107a), LAMP-2 (CD107b), and LAMP-3 (CD63). In order to detect these LAMP antibodies during degranulation, the LAMP antibodies must be added to the cell culture supernatant during the killing assay.

Materials needed:

NK cells and K562 cells
Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer
Flow tubes
Antibodies to LAMP proteins

Note: LAMP staining is performed at 37 °C. All other staining is carried out at room temperature

1. Set up killing assay for 2–4 h with desired effector (NK) to target (K562) cell ratios. Also set up wells with NK cells alone, and K562 cells alone as negative controls. Add LAMP antibodies at the recommended concentration during the killing assay.

2. Harvest cells, and wash cells two times to remove media: Add 2 mL flow cytometry staining buffer, vortex, and spin for 1 min (3100–3500 rpm/1049–1999 $\times g$) to 5 min (1250–1500 rpm/350–500 $\times g$) depending on the centrifuge used. Then block Fc receptors using human IgG and Mouse IgG for 10 min at room temperature. Do not wash out.
3. Surface stain for desired antibodies for 30 min at room temperature.
4. Wash cells two times in 2 mL flow cytometry staining buffer as above.
5. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

3 Intracellular Staining: Tips and Tricks

Intracellular staining involves more time as a fixation and permeabilization step are required. We will outline the various protocols for intracellular staining using the following fixation and permeabilization buffers: Formaldehyde/Saponin, the FoxP3/transcription factor buffer system, and Formaldehyde/Methanol. For more information on other intracellular staining protocols, please refer to the paper by Peter Krutzik and Garry Nolan (Cytometry A. Volume 55, Issue 2, 2003, Pages 61–70). As mentioned above, mouse and rat WB can be stained using these protocols. Refer to the modifications necessary in the surface staining section of this chapter.

There are a few key points to keep in mind when staining for intracellular or secreted proteins. Fixation can dim some surface markers, so if possible, surface marker staining should be carried out prior to fixation. In addition, order matters for intracellular staining. You **MUST** fix your cells *before* you permeabilize them; failure to do so will kill your cells (see Chap. 10 A for more information on permeabilizing your cells prior to fixation). With these suggestions in mind, you should be able to detect surface markers and intracellular proteins optimally.

3.1 Intracellular Staining: Formaldehyde/Saponin

3.1.1 Formaldehyde/Saponin IC Staining in WB with Unconjugated Antibodies

Materials needed:

1 $\times$ human lyse buffer

Human IgG block, mouse IgG block, or Fc block

Flow cytometry staining buffer

1 $\times$ PBS

1 $\times$ HBSS

1% Formaldehyde (dilute stock with 1 $\times$ PBS)

1 $\times$ Saponin (0.1% solution)

Flow tubes

Unconjugated IC antibody

Secondary antibody (e.g., Goat anti-mouse PE)

Costain antibodies (if needed)

Note: Carry out staining at room temperature

1 mL of unwashed WB will yield enough blood for approximately six tubes

1. Wash ~1 mL of WB with 2 mL HBSS, two times. This removes the plasma. Blood pellets are looser and can't be dumped out. Remove the plasma with a transfer pipette. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.
Note: For IC staining of WB, the RBCs need to be lysed before fixation occurs. If RBCs are fixed with formaldehyde, it creates a goopy mess.
2. Add 2–3 mL of 1 $\times$ human lyse buffer to each tube, and vortex each tube thoroughly to mix. This will lyse all of the red blood cells and leave only the white blood cells. Incubate for 10 min at room temperature in the dark, vortex each tube again, and incubate for an additional 10 min. The blood should turn from opaque to clear if the lysis is good. Once the cells have been lysed it is OK to dump the tubes after spinning. You no longer need to use a transfer pipette.
3. Spin the cells down and wash two to three times with 2 mL 1 $\times$ PBS. Spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.
4. Dilute 4% formaldehyde to 1% formaldehyde using 1 $\times$ PBS, then add to your cells (example: 300 μ L 1 $\times$ PBS and 100 μ L formaldehyde). Formaldehyde fixes the outer membrane of the cells. Incubate the cells in 1% formaldehyde for 10–15 min at room temperature.
5. Wash the cells once with 2 mL PBS to remove the excess 1% formaldehyde. Spin cells for 2 min (3100–3500 rpm/1049–1999 $\times$ g) to 10 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.
6. Wash once with 2 mL 1 $\times$ Saponin buffer (SAP). Saponin permeabilizes the cell membrane (creates holes in it) so that the antibody can reach the inside of the cell. Spin cells for 2 min (3100–3500 rpm/1049–1999 $\times$ g) to 10 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.
7. Block the blood using human IgG for 10 min at room temperature. Do not wash it out.
8. Resuspend cells in enough SAP for your experiment. Aliquot 100 μ L cells into each tube (e.g., if you have 8 tubes, resuspend the cells in at least 800 μ L SAP). There should be about 100,000–1,000,000 cells in each tube.
9. Add the primary antibody and incubate for 30 min at room temperature.
10. Wash cells two times with 2 mL SAP. Spin cells for 2 min (3100–3500 rpm/1049–1999 $\times$ g) to 10 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.
11. Add the secondary antibody and incubate for 20 min at room temperature in the dark.

12. Wash cells two times with 2 mL SAP. Spin cells for 2 min (3100–3500 rpm/1049–1999 $\times$ g) to 10 min (1250–1500 rpm/ 350–500 $\times$ g) depending on the centrifuge used.
13. If a costain is required, block prior to adding the costain antibody/antibodies. Fc block with the host of the species of antibody you are using. For example, if your costains are Mxsh, then use mouse IgG block. To do this, add mouse IgG for 10 min at room temperature, and do not wash it out. Then add the directly conjugated antibody/antibodies for 20 min. Then wash cells two times in 2 mL flow cytometry staining buffer. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used. If no costains are required, proceed to **step 14**.
14. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

3.1.2 Formaldehyde/Saponin IC Staining in WB with Directly Conjugated Antibodies

Materials needed:

- 1 $\times$ human lyse buffer
- Human IgG block, mouse IgG block, or Fc block
- Flow cytometry staining buffer
- 1 $\times$ PBS
- 1 $\times$ HBSS
- 1% formaldehyde (dilute stock with 1 $\times$ PBS)
- 1 $\times$ Saponin (0.1% solution)
- Flow tubes
- Directly conjugated antibodies (surface markers and IC)

Note: Carry out staining at room temperature

1 mL of unwashed WB will yield enough blood for approximately six tubes

1. Wash ~1 mL of WB with 2 mL HBSS, two times. This removes the plasma. Blood pellets are looser and can't be dumped out. Remove the plasma with a transfer pipette. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.

Note: For IC staining of WB, the RBCs need to be lysed before fixation occurs. If RBCs are fixed with Formaldehyde, it creates a goopy mess.

2. Add 2–3 mL of 1 $\times$ human lyse buffer to each tube, and vortex each tube thoroughly to mix. This will lyse all of the red blood cells and leave only the white blood cells. Incubate for 10 min at room temperature in the dark, vortex each tube again, and incubate for an additional 10 min. The blood should turn from opaque to clear if the lysis is good. Once the cells have been lysed it is OK to dump the tubes after spinning. You no longer need to use a transfer pipette.

3. Spin the cells down and wash two to three times with 2 mL 1× Flow cytometry staining buffer. If surface staining with costains is required, proceed to **step 4**. Otherwise, proceed to **step 6**.
4. Block cells with human and mouse IgG block for 10 min at room temperature.
5. Add each directly conjugated costain antibody and incubate at room temperature for 25 min.
6. Wash cells two times with 1× PBS. Spin cells for 1 min (3100–3500 rpm/1049–1999 × *g*) to 5 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.
7. Dilute 4% formaldehyde to 1% formaldehyde using 1× PBS, then add to your cells (example: 300 μL 1× PBS and 100 μL formaldehyde). Formaldehyde fixes the outer membrane of the cells. Incubate the cells in 1% formaldehyde for 10–15 min at room temperature.
8. Wash the cells 1× with 2 mL PBS to remove the excess 1% formaldehyde. Spin cells for 2 min (3100–3500 rpm/1049–1999 × *g*) to 10 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.
9. Wash once with 2 mL 1× Saponin buffer (SAP). Saponin permeabilizes the cell membrane (creates holes in it) so that the antibody can reach the inside of the cell. Spin cells for 2 min (3100–3500 rpm/1049–1999 × *g*) to 10 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.
10. Resuspend cells in enough SAP for your experiment. Aliquot 100 μL cells into each tube (e.g., if you have 8 tubes, resuspend the cells in at least 800 μL SAP). There should be about 100,000–1,000,000 cells in each tube.
11. Block the cells again with human IgG and incubate for 10 min at room temperature. Do not wash it out.
12. Add each directly conjugated IC antibody and incubate for 30 min at room temperature.
13. Wash cells once with 2 mL 1× SAP. Spin cells for 2 min (3100–3500 rpm/1049–1999 × *g*) to 10 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.
14. Resuspend cells in ~400 μL flow cytometry staining buffer for flow cytometry analysis.

3.1.3 Formaldehyde/Saponin IC Staining in PBMCs with Unconjugated Antibodies

Materials needed:

Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer
1× PBS
1× HBSS
1% formaldehyde (dilute stock with 1× PBS)
1× Saponin (0.1% solution)
Flow tubes

Unconjugated IC antibody

Secondary antibody (e.g., Goat anti-mouse PE)

Costain antibodies (if needed)

Note: Carry out staining at room temperature

1. Harvest the cells down and wash two times with 2 mL 1× PBS. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
2. Dilute 4% formaldehyde to 1% formaldehyde using 1× PBS, then add to your cells (example: 300 μL 1× PBS and 100 μL formaldehyde). Formaldehyde fixes the outer membrane of the cells. Incubate the cells in 1% formaldehyde for 10–15 min at room temperature.
3. Wash the cells once with 2 mL PBS to remove the excess 1% formaldehyde. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
4. Wash once with 2 mL 1× Saponin Buffer (SAP). Saponin permeabilizes the cell membrane (creates holes in it) so that the antibody can reach the inside of the cell. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
5. Block the blood using Human IgG for 10 min at room temperature. Do not wash it out.
6. Resuspend cells in enough SAP for your experiment. Aliquot 100 μL cells into each tube (e.g., if you have 8 tubes, resuspend the cells in at least 800 μL SAP). There should be about 100,000–1,000,000 cells in each tube.
7. Add the primary antibody and incubate for 30 min at room temperature.
8. Wash cells two times with 2 mL SAP. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
9. Add the secondary antibody and incubate for 20 min at room temperature.
10. Wash cells two times with 2 mL SAP. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
11. If a costain is required, block prior to adding the costain antibody/antibodies. Fc block with the host of the species of antibody you are using. For example, if your costains are Mxsh, then use mouse IgG block. To do this, add mouse IgG for 10 min at room temperature, and do not wash it out. Then add the directly conjugated antibody/antibodies for 20 min. Then wash cells two times in 2 mL flow cytometry staining buffer. Spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used. If no costains are required, proceed to **step 12**.
12. Resuspend cells in ~400 μL flow cytometry staining buffer for flow cytometry analysis.

3.1.4 Formaldehyde/Saponin IC Staining in PBMCs with Directly Conjugated Antibodies

Materials needed:

Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer
1× PBS
1× HBSS
1% formaldehyde (dilute stock with 1× PBS)
1× Saponin (0.1% solution)
Flow tubes
Directly conjugated IC antibody
Costain antibodies (if needed)

Note: Carry out staining at room temperature

1. Harvest the cells down and wash two times with 2 mL flow cytometry staining buffer. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
2. Block cells with human IgG and mouse IgG block for 10 min at room temperature.
3. Surface stain with your costain antibodies for 25 min at room temperature.
4. Wash cells two times with 2 mL 1× PBS. Spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
5. Dilute 4% formaldehyde to 1% formaldehyde using 1× PBS, then add to your cells (example: 300 µL 1× PBS and 100 µL formaldehyde). Formaldehyde fixes the outer membrane of the cells. Incubate the cells in 1% formaldehyde for 10–15 min at room temperature.
6. Wash the cells once with 2 mL PBS to remove the excess 1% formaldehyde. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
7. Wash once with 2 mL 1× Saponin buffer (SAP). Saponin permeabilizes the cell membrane (creates holes in it) so that the antibody can reach the inside of the cell. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
8. Block cells again with human IgG and mouse IgG block for 10 min at room temperature.
9. Resuspend cells in enough SAP for your experiment. Aliquot 100 µL cells into each tube (e.g., if you have 8 tubes, resuspend the cells in at least 800 µL SAP). There should be about 100,000–1,000,000 cells in each tube.
10. Add the IC antibody and incubate for 30 min at room temperature.
11. Wash cells once with 2 mL SAP. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.

12. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

3.2 Intracellular Staining: FoxP3/Transcription Factor Buffer Set

3.2.1 FoxP3/Transcription Factor Buffer Staining in WB with Unconjugated Antibodies

Materials needed:

- 1 $\times$ Human Lyse
- Human IgG block, Mouse IgG block, or Fc block
- Flow cytometry staining buffer
- 1 $\times$ PBS
- 4 $\times$ FoxP3 Fix concentrate
- 1 $\times$ FoxP3 Diluent
- 10 $\times$ FoxP3 Perm buffer
- Flow tubes
- Unconjugated IC antibody
- Secondary antibody (eg, Goat anti-rabbit PE)
- Costain antibodies (if needed)

Note: Carry out staining at 4 $^{\circ}$ C

1 mL of unwashed WB will yield enough blood for approximately six tubes

1. Wash ~1 mL of WB with 2 mL HBSS, two times. This removes the plasma. Blood pellets are looser and can't be dumped out. Remove the plasma with a transfer pipette. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.

Note: For IC staining of WB, the RBCs need to be lysed before fixation occurs. If RBCs are fixed first, it creates a goopy mess.

2. Add 2–3 mL of 1 $\times$ Human Lyse to each tube, and vortex each tube thoroughly to mix. This will lyse all of the red blood cells and leave only the white blood cells. Incubate for 10 min at room temperature in the dark, vortex each tube again, and incubate for an additional 10 min. The blood should turn from opaque to clear if the lysis is good. Once the cells have been lysed it is ok to dump the tubes after spinning. You no longer need to use a transfer pipette.
3. Spin the cells down and wash two times with 2 mL 1 $\times$ PBS. Wash cells a third time with 2 mL *ice cold* 1 $\times$ PBS. Spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used. During washes, make up fresh 1 $\times$ Fixation Buffer by diluting 4 $\times$ Fixation Concentrate with Fixation Diluent (i.e., 100 μ L Fixation Concentrate + 300 μ L Fixation Diluent).

4. Resuspend cells in freshly made 1× Fixation Buffer using 0.4 mL/tube. Incubate at 4 °C for 30 min. During this incubation, make up 1× Permeabilization/Wash Buffer by diluting 10× Permeabilization/Wash Buffer with distilled water (i.e., 100 µL Permeabilization/Wash Buffer + 900 µL diH₂O) and keep at 4 °C.
5. Wash cells two times with freshly made, cold 1× Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
6. Block cells with Human IgG block for 10 min at room temperature.
7. Add the unconjugated IC antibody to cells and incubate for 30 min at 4 °C.
8. Wash cells once with cold 1× Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
9. Add the secondary antibody and incubate for 20 min at 4 °C.
10. If a costain is required, block prior to adding the costain antibody/antibodies. Fc block with the host of the species of antibody you are using. For example, if your costains are Mxsh, then use Mouse IgG block. To do this, add Mouse IgG for 10 min at room temperature, and do not wash it out. Then add the directly conjugated antibody/antibodies for 20 min. Then wash cells two times in 2 mL Flow cytometry staining buffer. Spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used. If no costains are required, proceed to **step 10**.
11. Resuspend cells in ~400 µL Flow cytometry staining buffer for flow cytometry analysis.

3.2.2 FoxP3/Transcription Factor Buffer Staining in WB with Directly Conjugated Antibodies

Materials needed:

- 1× human lyse buffer
- Human IgG block, mouse IgG block, or Fc block
- Flow cytometry staining buffer
- 1× PBS
- 4× FoxP3 Fixation Concentrate
- 1× FoxP3 Diluent
- 10× FoxP3 Permeabilization buffer
- Flow tubes
- Direct conjugate IC antibody
- Costain antibodies (if needed)

Note: Carry out staining at 4 °C

1 mL of unwashed WB will yield enough blood for approximately six tubes

1. Wash ~1 mL of WB with 2 mL HBSS, two times. This removes the plasma. Blood pellets are looser and can't be dumped out. Remove the plasma with a transfer pipette. For washes, spin cells for 1 min (3100–3500 rpm/1049–

1999 $\times g$) to 5 min (1250–1500 rpm/350–500 $\times g$) depending on the centrifuge used.

Note: For IC staining of WB, the RBCs need to be lysed before fixation occurs. If RBCs are fixed first, it creates a goopy mess.

2. Add 2–3 mL of 1 $\times$ human lyse buffer to each tube, and vortex each tube thoroughly to mix. This will lyse all of the red blood cells and leave only the white blood cells. Incubate for 10 min at room temperature in the dark, vortex each tube again, and incubate for an additional 10 min. The blood should turn from opaque to clear if the lysis is good. Once the cells have been lysed it is OK to dump the tubes after spinning. You no longer need to use a transfer pipette.
3. Block cells with human IgG and mouse IgG block for 10 min at room temperature.
4. Surface stain with directly conjugated antibodies for 25 min at room temperature.
5. Spin the cells down and wash two times with 2 mL *cold* 1 $\times$ PBS. Spin cells for 1 min (3100–3500 rpm/1049–1999 $\times g$) to 5 min (1250–1500 rpm/350–500 $\times g$) depending on the centrifuge used. During washes, make up fresh 1 $\times$ Fixation Buffer by diluting 4 $\times$ Fixation Concentrate with Fixation Diluent (i.e., 100 μ L Fixation Concentrate + 300 μ L Fixation Diluent).
6. Resuspend cells in freshly made 1 $\times$ Fixation Buffer using 0.4 mL/tube. Incubate at 4 $^{\circ}$ C for 30 min. During this incubation, make up 1 $\times$ Permeabilization/Wash Buffer by diluting 10 $\times$ Permeabilization/Wash Buffer with distilled water (i.e., 100 μ L Permeabilization/Wash Buffer + 900 μ L diH₂O) and keep at 4 $^{\circ}$ C.
7. Wash cells two times with freshly made, cold 1 $\times$ Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 $\times g$) to 10 min (1250–1500 rpm/350–500 $\times g$) depending on the centrifuge used.
8. Block cells again with human IgG and mouse IgG block for 10 min at room temperature.
9. Add the IC antibody to cells and incubate for 30 min at 4 $^{\circ}$ C.
10. Wash cells once with cold 1 $\times$ Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 $\times g$) to 10 min (1250–1500 rpm/350–500 $\times g$) depending on the centrifuge used.
11. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

3.2.3 FoxP3/Transcription Factor Buffer Staining in PBMCs with Unconjugated Antibodies

Materials needed:

Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer
1 $\times$ PBS
4 $\times$ FoxP3 Fixation Concentrate
1 $\times$ FoxP3 Diluent

10× FoxP3 Permeabilization Buffer

Flow tubes

Unconjugated IC antibody

Secondary antibody (e.g., Goat anti-rabbit PE)

Costain antibodies (if needed)

Note: Carry out staining at 4 °C

1 mL of unwashed WB will yield enough blood for ~6 tubes

1. Harvest cells and wash two times with 2 mL 1× PBS. Wash cells a third time with 2 mL *ice cold* 1× PBS. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used. During washes, make up fresh 1× Fixation Buffer by diluting 4× Fixation Concentrate with Fixation Diluent (i.e., 100 µL Fixation Concentrate + 300 µL Fixation Diluent).
2. Resuspend cells in freshly made 1× Fixation Buffer using 0.4 mL/tube. Incubate at 4 °C for 30 min. During this incubation, make up 1× Permeabilization/Wash Buffer by diluting 10× Permeabilization/Wash Buffer with distilled water (i.e., 100 µL Permeabilization/Wash Buffer + 900 µL diH₂O) and keep at 4 °C.
3. Wash cells two times with freshly made, cold 1× Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
4. Block cells with human IgG block for 10 min at room temperature.
5. Add the IC antibody to cells and incubate for 30 min at 4 °C.
6. Wash cells once with cold 1× Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
7. Add the secondary antibody and incubate for 20 min at 4 °C.
8. If a costain is required, block prior to adding the costain antibody/antibodies. Fc block with the host of the species of antibody you are using. For example, if your costains are Mxsh, then use mouse IgG block. To do this, add mouse IgG for 10 min at room temperature, and do not wash it out. Then add the directly conjugated antibody/antibodies for 20 min. Then wash cells two times in 2 mL flow cytometry staining buffer. Spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used. If no costains are required, proceed to **step 9**.
9. Resuspend cells in ~400 µL flow cytometry staining buffer for flow cytometry analysis.

3.2.4 FoxP3/Transcription Factor Buffer Staining in PBMCs with Directly Conjugated Antibodies

Materials needed:

Human IgG block, mouse IgG block, or Fc block

Flow cytometry staining buffer

1× PBS

4× FoxP3 Fixation Concentrate
1× FoxP3 Diluent
10× FoxP3 Permeabilization Buffer
Flow tubes

Direct conjugate IC antibody
Costain antibodies (if needed)

Note: Carry out staining at 4 °C

1 mL of unwashed WB will yield enough blood for ~6 tubes

1. Harvest cells, wash two times with 2 mL flow cytometry staining buffer. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used. Block cells with human IgG and mouse IgG block for 10 min at room temperature.
2. Surface stain with directly conjugated antibodies (5–10 µL/sample) for 25 min at room temperature.
3. Spin the cells down and wash two times with 2 mL cold 1× PBS. Spin cells for 1 min (3100–3500 rpm/1049–1999 × g) to 5 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used. During washes, make up fresh 1× Fixation Buffer by diluting 4× Fixation Concentrate with Fixation Diluent (i.e., 100 µL Fixation Concentrate + 300 µL Fixation Diluent).
4. Resuspend cells in freshly made 1× Fixation Buffer using 0.4 mL/tube. Incubate at 4 °C for 30 min. During this incubation, make up 1× Permeabilization/Wash Buffer by diluting 10× Permeabilization/Wash Buffer with distilled water (i.e., 100 µL Permeabilization/Wash Buffer + 900 µL diH₂O) and keep at 4 °C.
5. Wash cells two times with freshly made, cold 1× Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
6. Block cells again with human IgG and mouse IgG block for 10 min at room temperature.
7. Add unconjugated FoxP3 antibody (or other IC antibody) to cells and incubate for 30 min at 4 °C.
8. Wash cells once with cold 1× Permeabilization/Wash Buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × g) to 10 min (1250–1500 rpm/350–500 × g) depending on the centrifuge used.
9. Resuspend cells in ~400 µL flow cytometry staining buffer for flow cytometry analysis.

3.3 Formaldehyde/Methanol Intracellular Staining

We do not recommend using formaldehyde/methanol to detect phosphoproteins in whole blood. With all the lysis steps and incubations that are required at room temperature, it is likely you will lose detection of your phospho-protein. Phospho-proteins are typically upregulated very quickly (usually within 5–20 min), and they

are stabilized by washing the cells in *ice cold* 1× PBS after the stimulation and prior to fixation. Processing blood on ice is simply not possible as the incubations need to be carried out at room temperature to minimize clumping of the WB. In addition, lysis will likely interfere with detection of phosphorylation.

3.3.1 Formaldehyde/Methanol Staining for Detection of Unconjugated Phospho-Proteins in PBMCs or Stimulated Cell Populations

Materials required:

1× human lyse
Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer
1× PBS (*ice cold* and room temperature)
4% formaldehyde
100% methanol
Flow tubes
Unconjugated phospho-antibody
Secondary antibody (eg, Goat anti-mouse PE)
Costain antibodies (if needed)

1. Harvest cells and wash two to three times with 2 mL *ice cold* 1× PBS. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 × *g*) to 5 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used. During washes, make up fresh 1× Fixation Buffer by diluting 4% formaldehyde with 1× PBS (i.e., 100 µL Fixation Concentrate + 300 µL 1× PBS).
2. Resuspend cells in freshly made 1× Fixation Buffer using 0.4 mL/tube. Incubate at room temperature for 10–15 min.
3. Wash cells two times with 2 mL flow cytometry staining buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × *g*) to 10 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.
4. Permeabilize cells in 100% *ice cold* methanol (e.g., 900 µL 100% methanol + 100 µL cells) for 30 min on ice or at 4 °C. There is also the option of storing cells at –20 °C at this point. Parafilm® the tubes if this is desired; cells can be stored in methanol for up to 5 weeks at –20 °C.
5. Wash cells two times with 2 mL 1× HBSS and then once with 2 mL flow cytometry staining buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 × *g*) to 10 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.
6. Block cells with human IgG block for 10 min at room temperature.
7. Add the unconjugated phospho antibody to cells and incubate for 30 min at room temperature.
8. Wash cells once with flow cytometry staining buffer. Spin cells for 1 minute (3100–3500 rpm/1049–1999 × *g*) to 5 min (1250–1500 rpm/350–500 × *g*) depending on the centrifuge used.

9. Add the secondary antibody and incubate for 20 min at room temperature in the dark. Wash cells once with flow cytometry staining buffer.
10. If a costain is required, block prior to adding the costain antibody/antibodies. Fc block with the host of the species of antibody you are using. For example, if your costains are Mxsh, then use mouse IgG block. To do this, add mouse IgG for 10 min at room temperature, and do not wash it out. Then add the directly conjugated antibody/antibodies for 20 min at room temperature.
11. Wash cells two times in 2 mL flow cytometry staining buffer. Spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used. If no costains are required, proceed to **step 12**.
12. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

3.3.2 Formaldehyde/Methanol Staining for Detection of Directly Conjugated Phospho-Proteins in PBMCs or Stimulated Cell Populations

Materials required:

1 $\times$ human lyse buffer
Human IgG block, mouse IgG block, or Fc block
Flow cytometry staining buffer
1 $\times$ PBS (*ice cold* and room temperature)
4% formaldehyde
100% methanol
Flow tubes
Directly conjugated phospho-antibody
Costain antibodies (if needed)

1. Harvest cells and wash two to three times with 2 mL *ice cold* 1 $\times$ PBS. For washes, spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used. During washes, make up fresh 1 $\times$ Fixation Buffer by diluting 4% formaldehyde with 1 $\times$ PBS (i.e., 100 μ L Fixation Concentrate + 300 μ L 1 $\times$ PBS).
2. Resuspend cells in freshly made 1 $\times$ Fixation Buffer using 0.4 mL/tube. Incubate at room temperature for 10–15 min.
3. Wash cells two times with 2 mL flow cytometry staining buffer. Spin cells for 2 min (3100–3500 rpm/1049–1999 $\times$ g) to 10 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.
4. Permeabilize cells in 100% *ice cold* methanol (e.g., 900 μ L 100% methanol + 100 μ L cells) for 30 min on ice or at 4 $^{\circ}$ C. There is also the option of storing cells at –20 $^{\circ}$ C at this point. Parafilm[®] the tubes if this is desired; cells can be stored in methanol for up to 5 weeks at –20 $^{\circ}$ C.
5. Wash cells two times with 2 mL 1 $\times$ HBSS and then once with 2 mL flow cytometry staining buffer. Spin cells for 1 min (3100–3500 rpm/1049–1999 $\times$ g) to 5 min (1250–1500 rpm/350–500 $\times$ g) depending on the centrifuge used.

6. Block cells with human IgG block and mouse IgG block for 10 min at room temperature.
7. Add the directly conjugated phospho antibody to cells and incubate for 30 min at room temperature. If you have costains, you may also add those antibodies at the same time.
8. Wash cells once with flow cytometry staining buffer.
9. Resuspend cells in ~400 μ L flow cytometry staining buffer for flow cytometry analysis.

Chapter 10

Troubleshooting



Christopher Hammerbeck and Christine Goetz

1 What Happens If I Permeabilize My Cells Before Fixation? What Happens If I Forget to Dilute My Perm Buffer to 1×?

If you permeabilize your cells prior to fixation, you will kill a lot of them. As we discussed in Chap. 7, permeabilization using various buffers pokes holes into the cell membrane to allow access of the intracellular antibodies. Fixation preserves proteins in their native state by cross-linking them to the membrane. When you permeabilize your cells prior to fixation, the perm buffers create holes in the cell membrane causing the cells to die. In Fig. 10.1a (right set of dot plots), we permmed and then immediately washed human PBMCs with 1× PBS to minimize the damage. Naïve and stimulated cells alike are sensitive to this and most of the cells shift to the right, indicating they are dying (low FSC). Notice how the live cell population is preserved when you Fix, and then permeabilize your cells which is the correct order (Fig. 10.1a, left set of dot plots).

Fixing and perming your cells in the wrong order pretty much ruins your experiment. What about using Saponin at 10× the recommended concentration? Consider how the IL-8 staining pattern changes using Saponin at 1× (recommended dilution) versus 10× (stock concentration). The middle dot plot in Fig. 10.1b illustrates the characteristic smear expected for IL-8 expression on monocytes stimulated with Monensin for 3 h. In this case, the correct concentration of SAP was used or 1× SAP. However, when 10× SAP is used, IL-8 expression has high background and all the cells look like they make IL-8 which we know isn't the case when the correct concentration of SAP is used.

C. Hammerbeck · C. Goetz (✉)

Department of Antibody Development, R&D Systems/Bio-Techne, Minneapolis, MN, USA
e-mail: Chris.Hammerbeck@bio-technie.com; Christine.Goetz@bio-technie.com

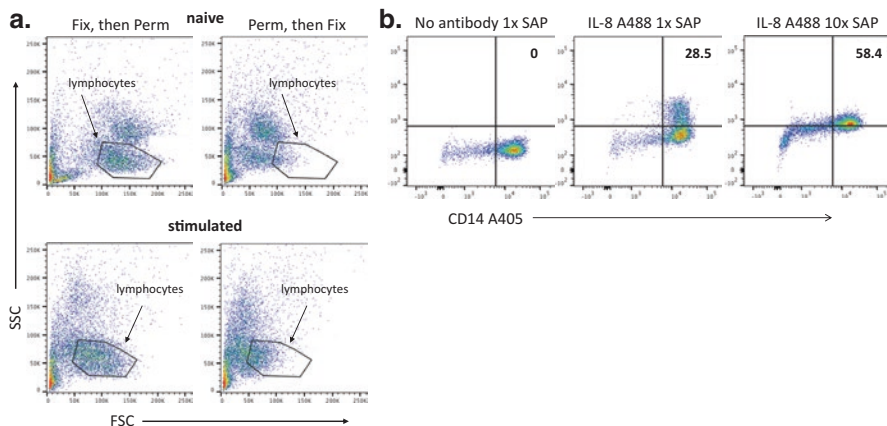


Fig. 10.1 Order of fixation and permeabilization and importance of diluting perm buffers. (a) Human PBMCs were either left untreated (naive; top panels) or stimulated with PMA (50 ng/mL) and Ionomycin (200 ng/mL) overnight (bottom panels). The correct order for fixation and permeabilization is shown in the left columns; FSC/SSC profiles are shown for the naive and stimulated lymphocyte populations. The FSC/SSC profiles in the right columns show the effect of perming 1st, following by fixation. The cells were run on a BD LSRFortessa™ and analyzed using FlowJo® software. (b) Human monocytes were stimulated with Monensin (50 ng/mL) for 3 h. Cells were harvested and surface stained for hCD14 A405 and fixed and permred using 1% Formaldehyde and either 1x (left and middle dot plot) or 10x SAP (right dot plot). The cells were then stained intracellularly with no antibody (left dot plot) or hIL-8 A488. The stained cells were run on a BD LSRFortessa™ and analyzed using FlowJo® software

2 What Happens If I Use PE or APC-Conjugated Surface Markers Prior to Permeabilization with Methanol?

Surface markers conjugated to either PE or APC will be dimmed or will completely lose signal after permeabilization with Methanol. The drawback to treating cells with methanol is that not all fluorochromes are compatible with methanol treatment. This is especially true for large fluorochromes such as PE, PerCP, and APC. Methanol significantly reduces, or in some cases completely abolishes fluorescence by these fluorochromes. In Fig. 10.2 we see monocytes surface stained HLA-DR FITC (Fig. 10.2a), HLA-A PE (Fig. 10.2b) representing a highly expressed surface antigen and CD86 PE (Fig. 10.2c) representing a surface antigen that is expressed at low to intermediate levels. When monocytes are stained with these antibodies and are then fixed with 1% Formaldehyde and permeabilized with methanol, we can see the results by looking at the blue histograms. Now compare these same monocytes after being fixed and permeabilized using a standard FoxP3 fix and perm buffer system by looking at the green histograms. The staining for HLA-DR FITC remains unchanged but notice how much lower the expression levels are when using an HLA-A and CD86 PE antibody. HLA-A can still be detected because it is expressed at very high levels. Still, the level of expression following methanol treatment is

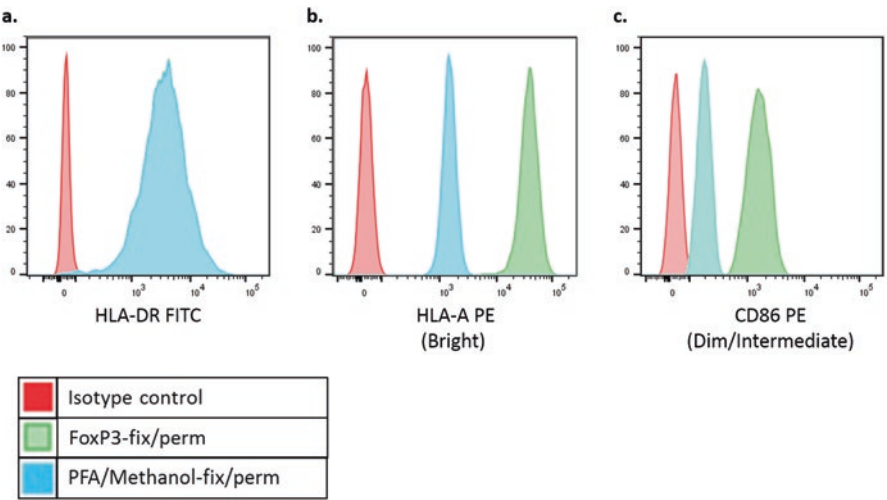


Fig. 10.2 Effects of methanol treatment on PE fluorescence. Human PBMCs were stained with CD14 A405 and combinations of HLA-DR FITC, HLA-A-PE, and CD86 PE or appropriate isotype control antibodies for 30 min at 4 °C. Cells were then fixed and permeabilized with either FoxP3 fix and perm buffers (15 min at 4 °C or 1% paraformaldehyde (10 min at room temperature) and 90% methanol. The expression of HLA-DR (a), HLA-A (b), and CD86 (c) is shown on CD14⁺ monocytes

nearly half that of cells treated with the FoxP3 buffer. For a protein that is not expressed as highly, such as CD86, the level of expression following methanol treatment is only slightly above isotype control background staining. A list of methanol-sensitive fluorochromes can be found in Table 9.1. **Fluorochromes sensitive and resistant to Methanol** that should be avoided on surface stains prior to 1% Formaldehyde fixation and permeabilization with Methanol. Colors such as PE, APC, and PerCP can still be used on intracellular phospho-specific antibodies AFTER methanol treatment but should not be used on surface antibodies that will be exposed to methanol treatment.

3 What Happens If I Stain My Cells with the Same Antibody Used for Stimulation?

You won't be able to detect expression of that antibody. Consider anti-CD3 as an example. MsxhCD3 (clone UCHT1) is a common antibody used to activate T cells and is typically used in combination with anti-CD28 for optimal costimulation. After activating cells with anti-CD3/anti-CD28, common surface markers to assess for cell activation include CD25 and CD69. Occasionally researchers will want to look at CD3 levels in conjunction with these activation markers. This becomes problematic when staining your cells with the same clone of anti-CD3 that you

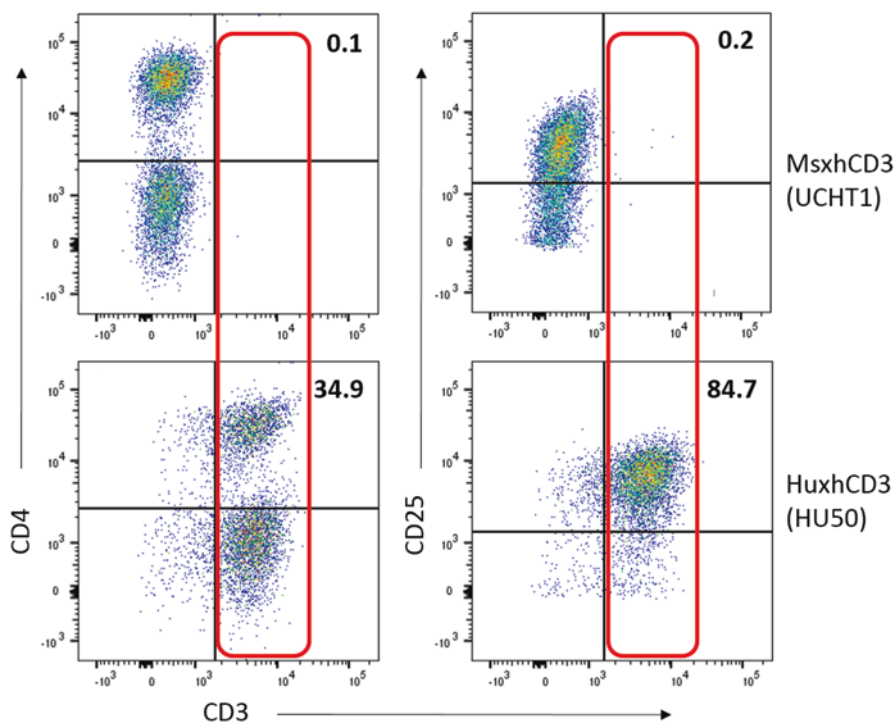


Fig. 10.3 Staining with the same clone used for stimulation. Human CD3⁺ T cells were purified and then cultured for 5 days in the presence of soluble MxshCD3 (UCHT1; 5 μ g/mL)/HuxhCD28 (HU51; 2 μ g/mL) or HuxhCD3 (HU50; 5 μ g/mL) and HuxhCD28 (HU51; 2 μ g/mL). The UCHT1 clone used in the top panels for stimulation is the same clone as the anti-CD3 flow antibody. The bottom panel shows cells stimulated with the HU50 anti-hCD3 antibody, and CD3 is readily detected using the anti-CD3 flow antibody (UCHT1 clone) because it is not the same clone. Cells were harvested and stained for CD3, CD4, and CD25. The stained cells were run on a BD LSRFortessa™ and analyzed using FlowJo® software. Live gates were set on total lymphocytes

activated your cells with. This is because you have *blocked* CD3 expression by using anti-CD3 on your cells prior to staining with the flow antibody.

Refer to Fig. 10.3. CD3⁺ T cells have been stimulated with soluble anti-CD3 (UCHT1 clone) and anti-CD28 for 5 days. These cells were then harvested and stained with the UCHT1 antibody conjugated to APC (MxshCD3 APC) along with CD4 and CD25. You will notice there is very low expression of CD3, which raises a red flag as CD3⁺ T cells were placed into culture so CD3 expression levels should be high. However, if you use a different clone of anti-CD3 to stimulate your cells (HU50), you can optimally detect CD3 expression levels using MxshCD3 APC (UCHT1 clone) because you haven't blocked expression of CD3 using the same clone you're trying to detect with.

4 How Can I Avoid Messy Staining with Costains and Secondary Antibodies of the Same Species?

There is a simple fix to this problem: block after you're done staining with your secondary antibody and prior to adding your costains. Consider the example shown in Fig. 10.4a. PBMCs were stained with a MsxhCD19 unconjugated antibody, and the secondary used was Goat-anti mouse PE or GAM PE. Mouse block was then added with the cells, followed by a MsxhCD3 FITC costain. The key step here is that Mouse block was added *after* the secondary and *prior* to the costain. As you can see, the staining pattern is clean and CD3 and CD19 expression are mutually exclusive as they are supposed to be (bottom dot plot). CD3 is directly above the double negative population, and CD19 is directly across from the double negative population, forming a right angle. Now the upper dot plot has a problem. The stain was carried out in the same order as the lower dot plot, but *no* Mouse block was used between the secondary and costain. It appears that CD3 is being expressed at intermediate levels on B cells and the data looks like a mess. We know this isn't possible based on published literature. Compensation isn't an issue, as the bottom dot plot looks fine. CD3 is specific for T cells, and CD19 is specific for B cells, so we shouldn't see intermediate levels of CD19 on T cells or CD3 expression on B cells. CD19 is unconjugated so you need to use a secondary antibody for detection. Since CD19 is mouse anti-human, goat-anti mouse PE can be used to detect CD19 expression.

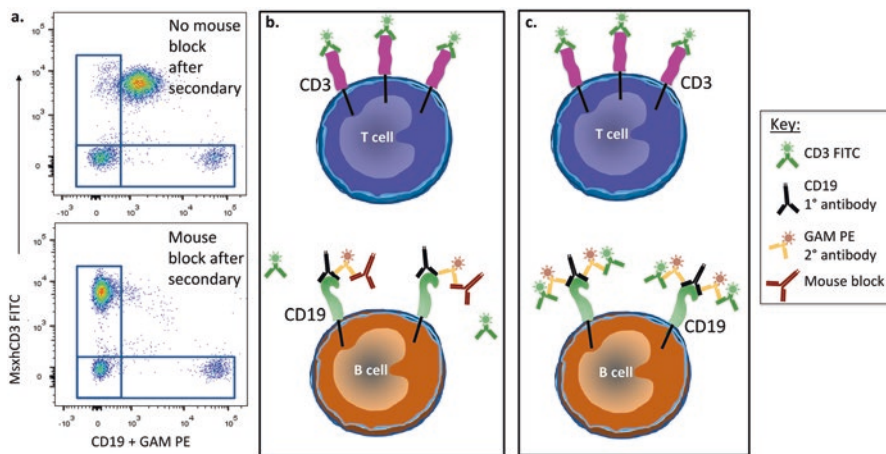


Fig. 10.4 Surface staining cells with costains and secondary antibodies of the same species. (a) Human PBMCs were blocked with Human IgG, and then stained with MsxhCD19. Goat anti-mouse PE (GAM PE) was used to detect MsxhCD19. Cells were then stained with MsxhCD3 FITC (top flow plot) or blocked with Mouse IgG, and then stained with MsxhCD3 FITC (bottom flow plot). The stained cells were run on a BD LSRFortessa™ and analyzed using Flowjo® software (gate was set on lymphocytes). (b) Schematic illustrating what happens when you block between the secondary antibody (GAM PE) and costain (MxhCD3 FITC). (c) Schematic illustrating what happens when you don't block between the secondary antibody (GAM PE) and costain (MxhCD3 FITC). See the text for details

Refer to the schematic in Fig. 10.4b to visualize how this works. You can avoid messy looking data if you block using Mouse IgG block *after* staining for the secondary and *before* you add the costain, or MxshCD3 FITC. There is no wash required between adding the Mouse block and MxshCD3 costain. In this example, Mouse block is required as the anti-hCD3 antibody is made in mouse cells. After using Mouse block, CD3 is unable to bind nonspecifically as shown by the free CD3 floating around (refer to Fig. 10.4b). If Mouse block isn't added between the secondary (GAM PE) and CD3 costain, CD3 can bind to the GAM PE secondary (refer to Fig. 10.4c). The GAM secondary is specifically binding to the Fc portion of the MxshCD3 FITC antibody. That is the problem occurring in that top dot plot which causes the data to look messy and uncompensated.

5 Why I Am Not Seeing NK1.1 Expression on Balb/c Mouse Splenocytes? Is CD14 Universally Expressed in Human and Mouse Monocytes? How About Other T and B Lineage Subsets?

NK1.1 is a strain-specific antibody for C57Bl/6 mice. As shown in Fig. 10.5a, NK1.1 expression is absent in Balb/c mouse WB but is highly expressed on C57Bl/6 mouse WB (gates were set using isotype controls). Human and mouse lineage markers such as CD3, CD4, CD19, and B220 are relatively similar in their expression pattern (see Fig. 10.5b). The ratio of CD3⁺ CD4⁺ T cells in human WB is higher than in mouse WB, but different mouse strains can also vary in their ratio of CD4⁺ to CD8⁺ T cells,

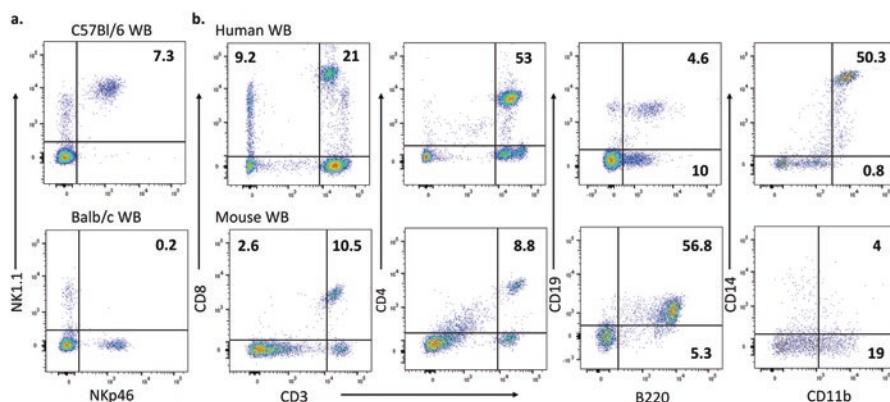


Fig. 10.5 Strain-specific antibodies and lack of CD14 expression in mouse monocytes. (a) C57Bl/6 and Balb/c mouse splenocytes were stained with NKp46 and NK1.1. The stained cells were run on a BD LSRFortessa™ and analyzed using Flowjo® software. (b) Human (top panel) and C57Bl/6 mouse (bottom panels) whole blood was stained with species-specific antibodies for the following: CD3, CD8, CD4, B220, CD19, CD11b, and CD14. The stained cells were run on a BD LSRFortessa™ and analyzed using FlowJo® software. Gates were set using isotype controls (data not shown)

so this isn't totally unexpected. Another apparent difference is that NK cells are detected in human WB (CD3⁻CD8⁺ population). However, no NK cells are detectable in mouse CD3⁻CD8⁺ cells. Turning to the monocyte population, we can see high expression of CD11b in both species. However, CD14 is virtually absent in mouse WB monocytes whereas CD14 is highly expressed in human WB monocytes. Thus, there are some differences in mouse and humans for certain lineage markers.

6 What Happens If You Use a FITC or A488 Antibody on GFP-Tagged Cells?

You will not be able to detect the FITC/A488-conjugated antibody on eGFP-tagged cells as FITC/A488 and eGFP are in the same channel (share the same PMT). As you can see in Fig. 10.6 in the first panel of dot plots, CD3 A488 is not detectable

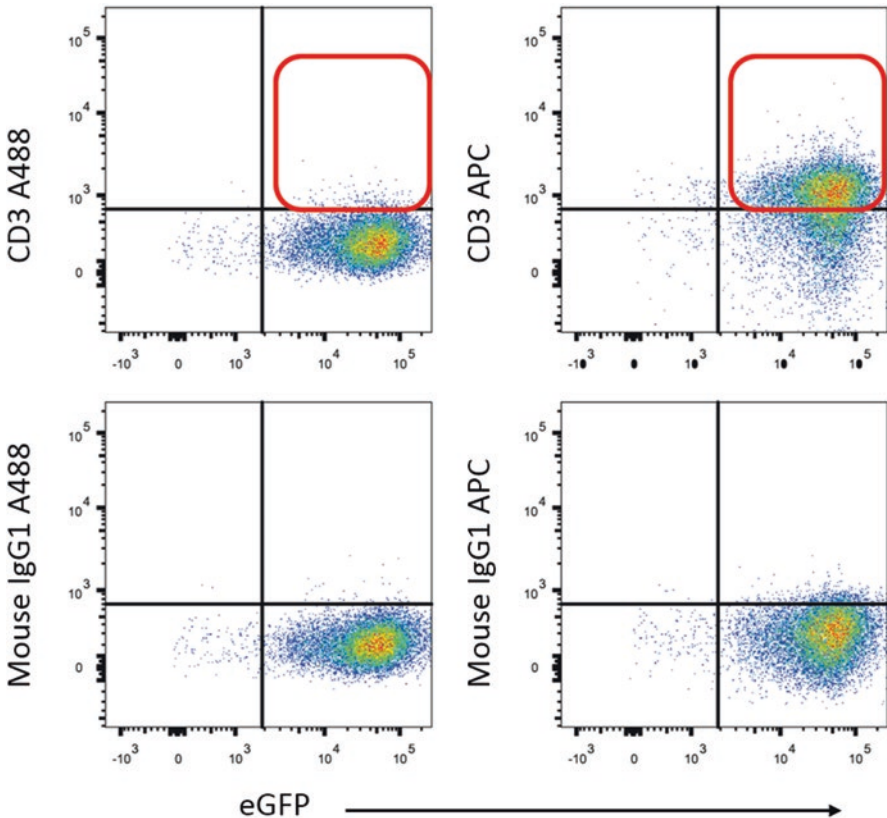


Fig. 10.6 CD3 A488 is not detected on Human CD3 HEK eGFP transfectants. hCD3 HEK eGFP transfectants were stained with the following: Mouse IgG1 A488, MxhCD3 A488, Mouse IgG1 APC, or MxhCD3 APC. The stained cells were run on a BD LSRFortessa™ and analyzed using FlowJo® software

because of the complete overlap with CD3 eGFP. CD3 A488 expression looks the same as the isotype. In the second panel of dot plots, you can see co-expression of CD3 APC on the CD3 eGFP transfectants. This is because CD3 APC isn't in the same channel as eGFP.

7 Should I Use FcR Block on All Cells and with All Antibodies?

The goal of blocking Fc receptors is to minimize the effects of non-antigen-specific binding of flow cytometry antibodies to cells via Fc receptors. When this happens, higher background staining with certain antibody isotypes (e.g., IgG2a) can occur, artificially enhancing the fluorescence in a given channel. Many cell types express Fc receptors; a summary can be found in Table 7.3 in Chap. 7. Consequently, this means that many cell types have the potential to bind antibodies in a non-antigen-specific manner so by blocking all cells, we reduce that risk. That said, some cell types are more problematic than others. Most flow cytometry antibodies are of the Ig gamma class (IgG); therefore, we are most concerned about blocking Fc gamma receptors which are most commonly found on innate immune cells in humans such as NK cells, monocytes, and neutrophils with lesser expression on B lymphocytes. TCR α/β T cells are known to express CD23 which bind to Ig epsilon (IgE) class antibodies but no flow cytometry antibodies are made using an IgE isotype so you could get away without blocking Fc receptors when staining these cells. TCR γ/δ T cells can express CD16a which bind to Ig gamma (IgG) class antibodies so blocking Fc receptors on these cells would be appropriate. Keep in mind that CD16 and CD32 have a low affinity for IgG antibodies and that the bigger concern is CD64 which can bind monomeric IgG (e.g., flow cytometry antibodies) and have an affinity for Mouse IgG2a antibodies. The trade-off to blocking Fc receptors with an Fc block reagent is that some Fc block reagents can interfere with the ability to subsequently detect Fc receptors on cells.

Consider the example in Fig. 10.7 where we are showing staining of Fc receptors on human monocytes. The Fc block reagent being used is a combination of anti-CD16/32/64 antibodies. Human monocytes express CD64 which binds to Mouse IgG2a antibodies. On unblocked cells we see that the background fluorescence using a Mouse IgG2a PE isotype control antibody is higher than when the monocytes are pretreated with either soluble human IgG or Fc block (Fig. 10.7a). However, we lose all fluorescence signals for CD16, CD32, and CD64 on monocytes that have been treated with Fc block (Fig. 10.7b). In other words, the Fc block reagents block the binding of the CD16, CD32, and CD64 antibodies to the CD16, CD32, and CD64 Fc receptors. In this case, blocking with soluble human IgG would be appropriate; it still reduces the background staining from the Mouse IgG2a isotype control antibody but preserves the detection of the CD16,

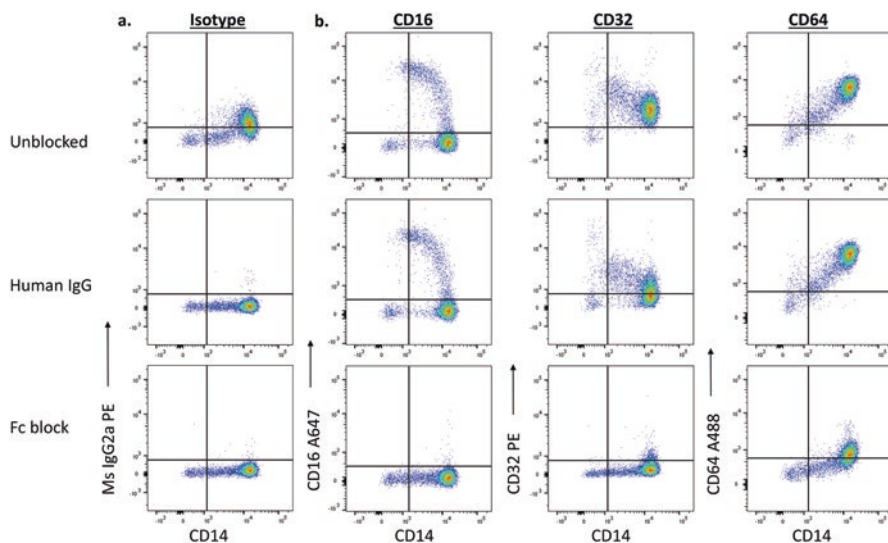


Fig. 10.7 Effects of blocking Fc receptors on the detection of Fc receptors by flow cytometry. Human PBMCs were left untreated or were incubated with human IgG or an Fc block cocktail consisting of mouse anti-human CD16, CD32, and CD64 antibodies for 15 min at 4 °C. Cells were then stained with CD14 A405, CD16 APC, CD32 PE, and CD64 A488 or appropriate isotype control antibodies for 30 min at 4 °C. Cells within the monocyte gate are shown. Background fluorescence using a Mouse IgG2a isotype control antibody is shown in (a). Staining for CD16, CD32, and CD64 is shown in (b)

CD32, and CD64 Fc receptors. This phenomenon is specific to the Fc receptors as staining for other surface antigens is unaffected when cells are pretreated with Fc block.

Depending on the cell type, a form of Fc block is necessary to reduce background fluorescence due to nonspecific antibody binding. The type of block (Fc block vs. soluble IgG) used needs to be determined by the cell type you are using and the surface antigens you are trying to detect.

8 Do I Need to Use PMA, Lonomycin, and Monensin (and/or Brefeldin A) for Detection of Secreted Cytokines in T Cells and Monocytes?

For detection of secreted cytokines in T cells, the answer is yes. As discussed in Chap. 7, addition of either Monensin or Brefeldin A to your cultures prior to intracellular cytokine staining is critical for detection of secreted cytokines [1]. Furthermore, T cells also require reactivation with PMA and Ionomycin for optimal

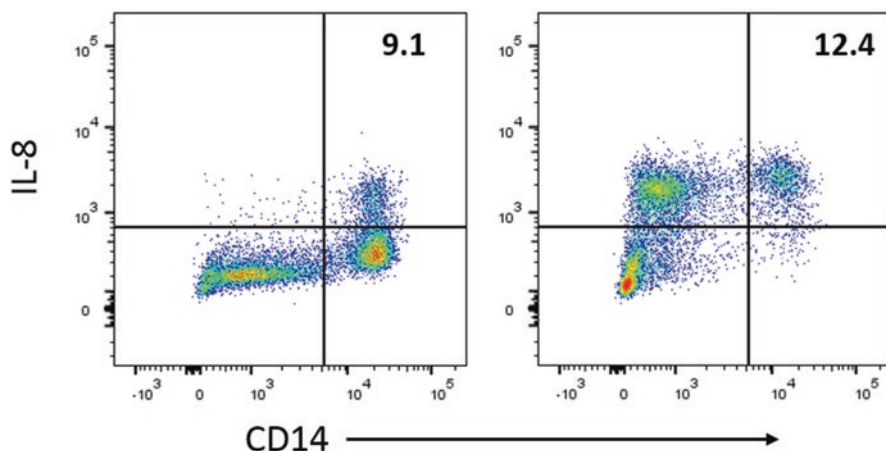


Fig. 10.8 Reactivation with PMA and Ionomycin is not necessary for detection of IL8 in monocytes. Human PBMCs were activated overnight with either Monensin (3 μ M) only (left panel) or LPS (1 μ g/mL)/Monensin (3 μ M; right panel). Cells were surface stained with CD14, fixed and permeated with 1% Formaldehyde/1 $\times$ SAP, and then stained intracellularly for IL-8. The stained cells were run on a BD LSRFortessa™ and analyzed using FlowJo® software. Monocytes were gated on and an isotype control was used to set gates for IL-8 (data not shown)

cytokine detection (refer to Fig. 7.32). In contrast, monocytes do not require inclusion of PMA and Ionomycin for detection of secreted cytokines. As shown in Fig. 10.8, IL-8 is readily detected with Monensin alone (~9.2%). However, activation with both LPS and Monensin induces a higher level of IL-8 expression (~12.4%) in CD14⁺ monocytes.

9 Can I Add My Secondary and Primary Antibody at the Same Time to Speed Up an Experiment?

You can add your primary and secondary antibodies at the same time but doing this will result in dimming of your primary antibody. This is because your primary and secondary antibodies will form a complex and competition will occur when binding to the cells. Refer to Fig. 10.9 and you can see that when the primary and secondary antibodies are added together, the signal is dimmed by about a log (blue histogram). In contrast, when the primary and secondary antibody stains are performed separately with a wash in between, there is optimal detection of your primary antibody (red histogram). The level of CD19 staining is also higher due to amplification of the signal by the secondary antibody.

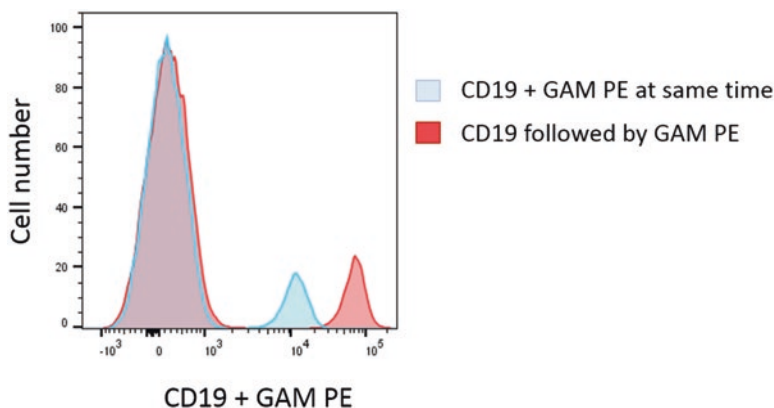


Fig. 10.9 Staining with primary and secondary antibodies at the same time. Human whole blood was stained with CD19 and GAM PE at the same time (blue histogram) or GAM PE was added separately after a wash (red histogram). The stained cells were run on a BD LSRFortessa™ and analyzed using FlowJo® software. Live gates were set on the lymphocyte population

10 Can Biotinylated Antibodies and the Streptavidin Secondary Be Added at the Same Time?

Historically, this is an issue with ELISA and IHC protocols which has carried over into many flow protocols. To test this directly, we compared whether a CD19 biotinylated antibody could be added directly with a streptavidin (SA) secondary reagent and if this affected the MFI of CD19 fluorescence or in any way compromised the quality of the flow plots. As shown in Fig. 10.10, when SA-PE is added subsequently to, or added simultaneously with, a biotinylated CD19 antibody, the detection of the CD19⁺ B cell population does not change. The percent of CD19⁺ cells in both conditions is the same, as is the MFI of PE fluorescence. This makes sense as the streptavidin secondary reagent only has specificity for the biotinylated CD19 antibody. This is where a streptavidin secondary differs from a traditional secondary antibody (e.g., goat anti-mouse, goat anti-rat, etc.) which will bind any antibody from a given species. The biggest concern with adding a biotinylated primary antibody and a streptavidin secondary at the same time is that the two might form a complex that may interfere with the ability of the primary antibody to bind its specific antigen. While combining the biotinylated CD19 antibody with a SA-PE secondary worked in the experiment in Fig. 10.10, we cannot guarantee this will work for every biotinylated antibody. We also cannot guarantee this will work when using a biotinylated antibody for the detection of intracellular antigens so be sure to test combinations of biotinylated antibodies and SA secondaries in your specific system.

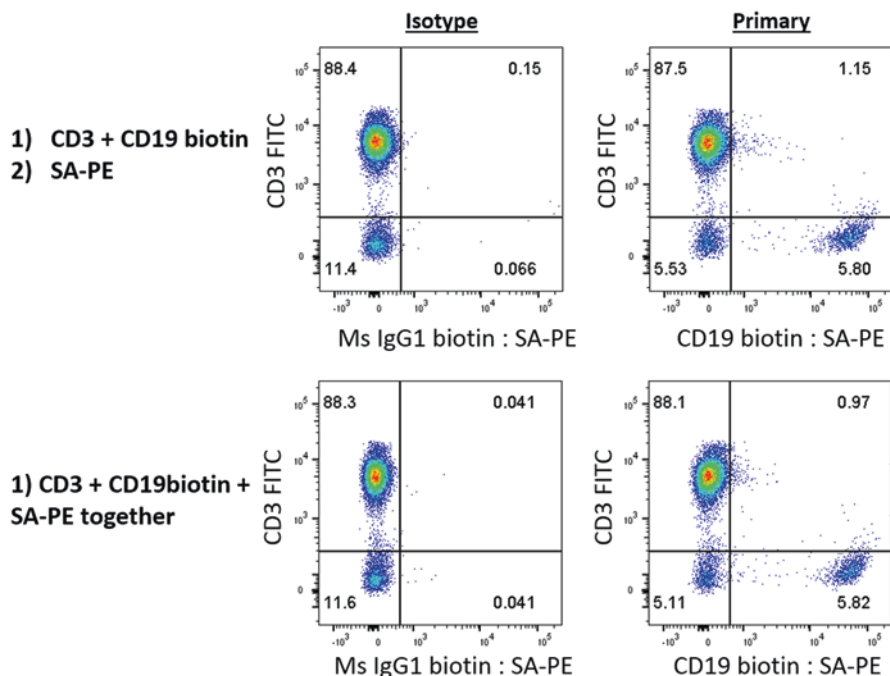


Fig. 10.10 Biotinylated antibody staining order. Human PBMC were stained with CD3 FITC and combinations of CD19 biotin and streptavidin (SA) PE or appropriate isotype control antibodies. In the top row, CD3 FITC and CD19 biotin or CD3 FITC and Ms IgG1 biotin were added first and incubated for 30 min at 4 °C. Cells were washed and then SA-PE was added for 30 min at 4 °C. In the bottom row, CD3 FITC, CD19 biotin and SA-PE or CD3 FITC, Ms IgG1 biotin and SA-PE were added simultaneously and incubated for 30 min at 4 °C

11 Does Volume Matter When Surface Staining Your Cells?

Most flow cytometry protocols recommend staining approximately 10^6 cells in 100 μ L because this is known to be effective, but there is room to vary these conditions. Some investigators like to stain cells in a smaller volume and for a shorter amount of time (such as 50 μ L for 15 min) with the theory being that as you decrease staining volume you increase antibody concentration and therefore decrease the amount of time necessary for the antibody to “find” and bind to the cell. We already know that antibodies bind very rapidly (Fig. 10.11) so in a small volume like 50 μ L, 15 min may be effective. It is impossible to foresee all the various combinations of volume, concentration, and time that might be relevant to a given experiment, so we would encourage you to test various combinations and find what works best for you given the principles that we’ve discussed throughout this book. To some extent, staining volume will be constrained by the concentration of the antibody you have purchased; if the vendor recommendation is to use 10 μ L per test, and you are using

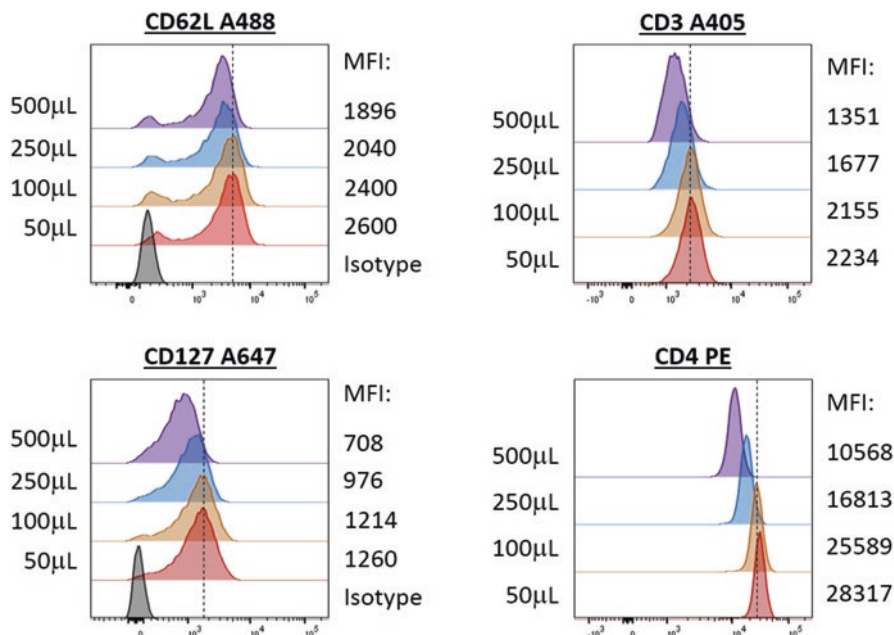


Fig. 10.11 Effects of staining volume on mean fluorescence intensity of antibodies. Human PBMCs were incubated with human IgG for 15 min at 4 °C and then stained with CD3 A405, CD4 PE, CD62L A488, and CD127 A647 or appropriate isotype control antibodies. Cells were stained in either 50 μ L, 100 μ L, 250 μ L, or 500 μ L. The MFI for each antibody was then determined. CD3⁺ cells were gated from all lymphocytes. CD4⁺ cells were gated from CD3⁺ cells only. CD127 and CD62L expression is shown for CD4⁺CD3⁺ cells

an 8-color panel, your staining volume would likely be around 80 μ L. However, as we've also discussed, you need to titrate your antibodies to determine if you need more, or less, of a given antibody. This will also affect your final staining volume. All else being equal, keep in mind that staining volume can affect the MFI of your stains. In Fig. 10.11, we are comparing the MFI of CD62L, CD127, CD4, and CD3 on T cells from PBMCs stained in a volume of 50 μ L, 100 μ L, 250 μ L, or 500 μ L. For the sake of simplicity, all cells were stained for 30 min. The MFI for each marker did not change dramatically across the various staining volumes and the pattern of expression for each marker was preserved. The black dashed line in each plot represents the peak expression for each marker when stained under the recommended conditions of 10^6 cells in 100 μ L for 30 min at 4 °C. Still, the MFI for cells stained in 500 μ L was almost 50% lower than the MFI for the same marker on cells stained at 50 μ L or 100 μ L. When using highly expressed lineage markers like CD3 and CD4 on bright colors such as PE, a 50% reduction in MFI won't be detrimental but a 50% reduction in the detection of moderately expressed proteins such as CD127 can be more problematic. Higher volumes for markers that are only weakly expressed would not be recommended as even a small decrease in MFI could dramatically affect your ability to detect those markers.

12 What Happens If I Can't Run My Cells on the Cytometer on the Same Day that I Stain Them?

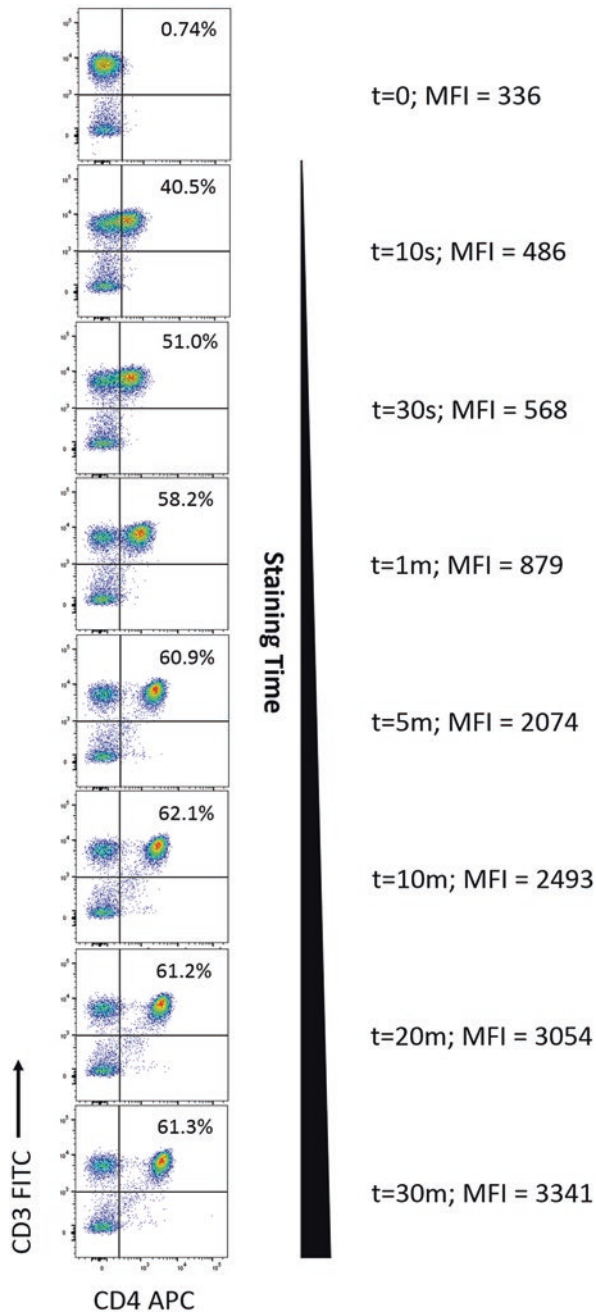
Generally, it's the best practice to run your cells on the same day that you process and stain them but there can be many reasons that this isn't possible. Most often, storing your cells covered at 4 °C won't result in a noticeable loss of signal; but in some cases, you may see a small decrease in the MFI of your proteins of interest. This will affect the detection of weakly expressed proteins more so than the detection of highly expressed proteins. The bigger concern can be a decrease in viability which could lead to fewer cells, or a disproportionate number of cells, in your live gate. Some investigators like to add an equal volume of a low percent PFA solution (0.2%–0.5%) to their cells to help avoid any loss of signal and to try and preserve the relative forward and side scatter profiles of their cells. A blanket statement cannot possibly cover all proteins of interest, all antibodies, and all fluorochromes. By virtue of the affinity for the specific antigen for which they are designed, flow cytometry antibodies have a high-binding avidity and a low off-rate so it's unlikely that the antibody will release from its antigen while sitting at 4 °C. Be careful when using tandem dyes (e.g., PE-Cy7 or PerCP-Cy5.5) which have historically had problems with stability. Newer tandem dyes are more stable than the first-generation tandem dyes and should be ok overnight if kept in the dark at 4 °C but longer-term storage of cells with older tandem dyes could be problematic.

The rules are a bit different if intracellular staining is involved. Once cells have been fixed, permeabilized, and stained intracellularly, you should run them on the same day as a more substantial loss of signal can occur over time. If fixed, permeabilized and intracellularly stained cells cannot be run on the same day, cells should be surface stained and fixed on day 1 and then permeabilized, stained intracellularly, and collected on a subsequent day. Cells fixed with 1% PFA should be stable if kept in staining buffer at 4 °C. In some cases, an extra fix step may be required before permeabilizing cells if fixing and permeabilization steps are carried out on subsequent days.

13 What Happens If I Accidentally Add the Wrong Antibody to My Tube? Can I Quickly Wash It Out and Reuse Those Cells?

If you accidentally added an isotype control antibody in place of a primary antigen-specific antibody, you should be ok. The isotype control antibody should not bind to your cells meaning that you can just add your antigen-specific primary antibody to the same tube. Unfortunately, if you added another antigen-specific primary antibody that is specific for an antigen expressed on the cells you are using, you'll likely have to start over. At play is the speed with which an antibody will bind its target antigen. For example, in Fig. 10.12 we are comparing how staining time affects the

Fig. 10.12 Effects of time on antibody binding. Human PBMCs were incubated with human IgG for 15 min at 4 °C and then stained with CD3 FITC for 30 min. Cells were washed and then stained with CD4 APC for 10 s, 30 s, 1 min, 5 min, 10 min, 20 min, or 30 min. The MFI of CD4 fluorescence and percent of CD4⁺ cells was then determined for each time point



detection of CD4⁺ T cells from human PBMCs. For the donor shown in Fig. 10.12, CD4⁺ T cells make up 61.3% of their lymphocyte repertoire. When stained for as little as 10 s, 40.5% of the lymphocyte population stained positive for CD4. Within 1 min, just under 60% of the lymphocytes stained positive for CD4 and by 5 min, we had detected the whole CD4⁺ T cell population. The MFI of CD4 expression increased the longer that the cells and antibody were allowed to incubate but by 1 min, a distinct CD4⁺ and CD4⁻ population had emerged.

14 Can Flow Cytometry Be Used to Count Cells?

Yes. In a roundabout way this was done during the NK cell killing assay in Fig. 7.26. The most common way to count cells using flow cytometry is to include reference beads in your flow cytometry samples. Reference beads are small polystyrene beads that are used at a known concentration. In many cases the beads are labeled with a dye that fluoresces when excited by a laser. Some beads only fluoresce in one channel while other beads fluoresce across multiple channels. The beads fluorescence often exceeds the fluorescence of stained cells so you typically don't need to worry about stained cells and reference beads occupying the same fluorescence decade. Reference beads often have a very low forward scatter and high-side scatter profile so they are visually distinct from most cell populations making it easy to distinguish the reference bead population from cells as seen in Fig. 10.13. When used, a defined volume of reference beads is added to your flow cytometry samples and a bead to

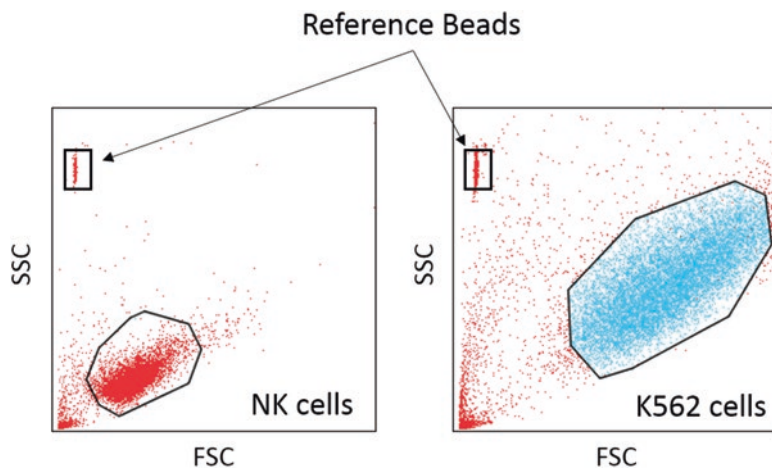


Fig. 10.13 Identification of reference counting beads in a mixed bead/cell population. 50 μ L of CountBright™ Absolute Counting Beads were added to 1×10^6 NK cells (left panel) or 1×10^6 K562 cells (right panel) in a total of 500 μ L of flow cytometry staining buffer. The samples were collected on a BD LSRFortessa™ and beads and cells were identified based on unique FSC and SSC properties

cell ratio is then determined. Since the reference bead concentration and sample volume are known, the cell concentration can be mathematically determined. The formula is usually something similar to the following:

$$\text{Cells/mL} = \frac{(\text{\# of cell events counted} \times \text{dilution factor})}{(\text{\# bead events counted} \times \text{dilution factor})} \times \text{\# beads/mL}$$

If 50 μL of cells, 50 μL of beads, and 200 μL of flow cytometry buffer were added to a sample tube, the total volume would be 300 μL . The dilution factor for both beads and cells would be 6 (300 μL total volume/50 μL beads or cells = 6).

15 Can the Method Used to Harvest Adherent Cells Affect the Ability to Detect Protein Expression by Flow Cytometry?

Adherent cells can be tricky as some of the proteins they express, especially those that are used to bind to the surface of a tissue culture flask, can be sensitive to enzymatic digestion. Trypsin, and trypsin-related products such as TrypLE Express, are proteolytic enzymes that break down proteins by cleaving the proteins at various amino acid sites, such as the carboxyl side of lysines and arginines [2]. This can be problematic if the antibody binding site on your protein of interest is on the portion of the protein that has been cleaved away. One option to work around this problem would be to gently scrape the adherent cells to release them using a rubber cell scraper. This should preserve protein expression. Some cells are more sensitive to manual scraping so this may not always be a viable solution. A second option would be to stain cells harvested by enzymatic digestion at a higher temperature, such as 37 °C. Staining at a higher temperature can allow the cells to reexpress any proteins that have been cleaved. A good example of this can be seen in Fig. 10.14. Human M2a macrophages were dissociated from a tissue culture plate using the TrypLE Express dissociation solution (Fig. 10.14, top panels) or by gentle scraping using a rubber cell scraper (Fig. 10.14, bottom panels). When macrophages treated with TrypLE Express were stained for CD11c at 4 °C, no CD11c expression was seen (Fig. 10.14, top left panel). By comparison, when macrophages were gently scraped off the tissue culture plate, CD11c expression was observed when staining was performed at either 4 °C or 37 °C (Fig. 10.14, bottom panels) suggesting that CD11c was cleaved by TrypLE Express. Staining TrypLE Express-treated macrophages at 37 °C improved CD11c staining by allowing CD11c to be reexpressed on macrophages (Fig. 10.14, top right panel).

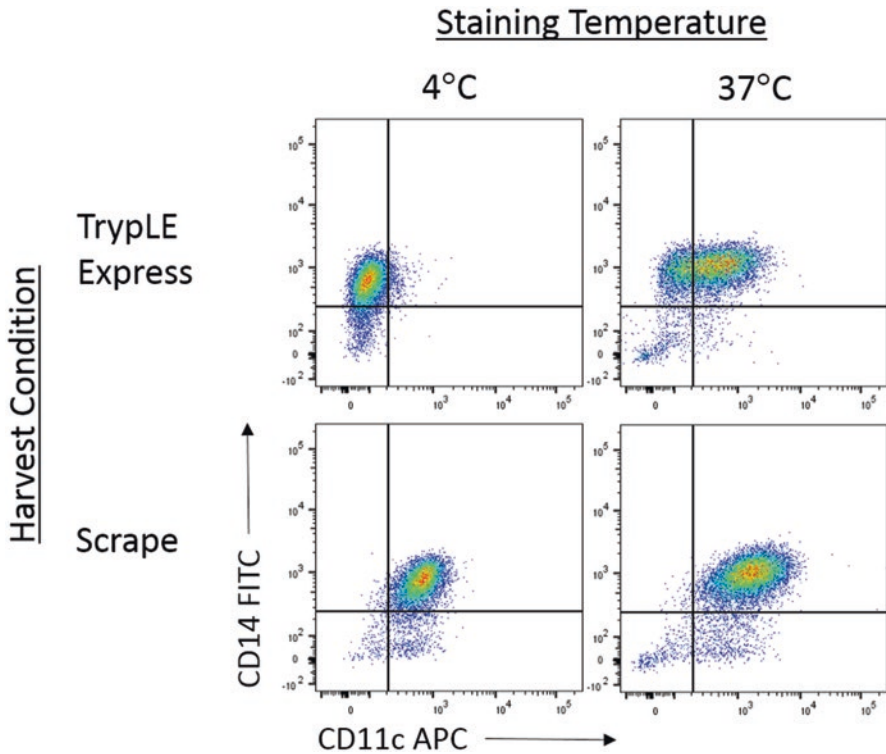


Fig. 10.14 Enzymatic dissociation prevents the detection of CD11c on human M2a macrophages. Human PBMCs were isolated from whole blood and CD14⁺ monocytes were isolated using a CD14⁺ monocyte MagCollect kit. Monocytes were cultured for 6 days in a 6-well plate with 50 ng/mL rhMCSF and then polarized to an M2a phenotype with 20 ng/mL IL-4 and 20 ng/mL IL-13 for 24 h. To harvest by enzymatic dissociation, plate wells were washed twice with PBS and 2 mL of TrypLE Express solution was added to the wells. The plate was then incubated at 37 °C for up to 30 min to release adherent cells. To harvest by scraping, plate wells were washed twice with PBS and 2 mL of PBS were added to the wells. Macrophages were then released by gently scraping the wells with a rubber cell scraper. Once harvested, the macrophages were labeled with a Violet viability dye to exclude dead cells, and surface stained for CD11c APC and CD14 FITC for 30 min at 4 °C or a combination of 15 min at 37 °C followed by 15 min at room temperature. The stained cells were then run on a BD LSRFortessa™ and analyzed using FlowJo® software

16 Materials

16.1 Flow Antibodies

- Mouse B220 (FAB1217; R&D Systems)
- Mouse CD3 (FAB4841; R&D Systems)
- Mouse CD4 (FAB554; R&D Systems)

- Mouse CD8 (FAB116; R&D Systems)
- Mouse CD11b (FAB1124; R&D Systems)
- Mouse CD14 (FAB982; R&D Systems)
- Mouse/Human CD19 (BD)
- Mouse NK1.1 (FAB8319; R&D Systems)
- Mouse NKp46 (FAB2225; R&D Systems)
- Human CD3 (FAB100; R&D Systems)
- Human CD3 FITC (FAB100F; R&D Systems)
- Human CD3 A488 (FAB100G; R&D Systems)
- Human CD3 APC (FAB100A; R&D Systems)
- Human CD3 A405 (FAB100V; R&D Systems)
- Human CD4 (FAB3791; R&D Systems)
- Human CD4 PE (FAB3791P; R&D Systems)
- Human CD11b (FAB16991; R&D Systems)
- Human CD14 (FAB3832; R&D Systems)
- Human CD14 A405 (FAB3832V; R&D Systems)
- Human CD16 APC (FAB2546A; R&D Systems)
- Human CD19 (FAB4867; R&D Systems)
- Human CD19 (MAB1509; R&D Systems)
- Human CD19 biotin (FAB4867B; R&D Systems)
- Human CD25 (FAB1020; R&D Systems)
- Human CD32 PE (FAB1330P; R&D Systems)
- Human CD62L A488 (FAB9787G; R&D Systems)
- Human CD64 A488 (FAB12571G; R&D Systems)
- Human CD86 PE (FAB141P; R&D Systems)
- Human CD127 A647 (FAB306R; R&D Systems)
- Human HLA-A PE (FAB7098P; R&D Systems)
- Human HLA-DR FITC (FAB4869F; R&D Systems)
- Human IL-8 (IC208; R&D Systems)
- Human IL-8 A488 (IC208G; R&D Systems)

16.2 Isotype Control Antibodies

- Mouse IgG1 A488 (IC002G; R&D Systems)
- Mouse IgG1 PE (IC002P; R&D Systems)
- Mouse IgG1 A647 (IC002R; R&D Systems)
- Mouse IgG1 APC (IC002A; R&D Systems)
- Mouse IgG1 biotin (IC002B; R&D Systems)
- Mouse IgG2a PE (IC003P; R&D Systems)

16.3 Secondary Antibodies and Staining Reagents

- Goat anti-mouse IgG (GAM) PE (F0102B; R&D Systems)
- Streptavidin PE (F0040; R&D Systems)

16.4 MISC Flow Cytometry Reagents

- CountBright™ Absolute Counting Beads (ThermoFisher Scientific)

16.5 Cell Culture Reagents

- TrypLE™ Express Enzyme (ThermoFisher Scientific)

16.6 Cell Activation Reagents

- MxshCD3 (MAB100; R&D Systems)
- PMA (Sigma)
- Ionomycin (Sigma)
- Monensin (Sigma)
- LPS (Sigma)
- Recombinant Human M-CSF (216-MC; R&D Systems)
- Recombinant Human IL-4 (204-IL; R&D Systems)
- Recombinant Human IL-13 (213-ILB; R&D Systems)

16.7 Cell Purification Kits

- MagCollect™ Human CD3+ T cell isolation kit (MAGH101; R&D Systems)
- MagCollect™ Human CD14+ cell isolation kit (MAGH105; R&D Systems)

16.8 Staining Buffers

- FlowX FoxP3/Transcription Factor Fixation & Perm Buffer Kit (FC012; R&D Systems)
- Normal Human IgG (1-001-A; R&D Systems)

- Mouse IgG
- Flow cytometry staining buffer (FC001; R&D Systems)
- 1× Fixation buffer (FC004; R&D Systems)
- 1× Saponin (FC005; R&D Systems)
- Flow Cytometry Human Lyse Buffer (FC002; R&D Systems)

References

1. Foster B, Prussin C, Liu F, Whitmire JK, Whitton JL. Detection of intracellular cytokines by flow cytometry. *Curr Protoc Immunol*. 2007;78:6.
2. Brown MA, Wallace CS, Anamelechi CC, Clermont E, Reichert WM, Truskey GA. The use of mild trypsinization conditions in the detachment of endothelial cells to promote subsequent endothelialization on synthetic surfaces. *Biomaterials*. 2007;28(27):3928–35.

Glossary

- Abort rate** Events that are discarded by the flow cytometer when samples are being acquired too quickly to allow discrimination of real cellular events versus debris.
- Activation marker** A protein that is expressed only after a cell is activated.
- Alexa Fluors** Small molecule dyes that are bright and photostable. They span from the UV to InfraRed light spectrum (ex. A350 to A750).
- Analog to Digital Converters (ADCs)** This component of the electronics system samples and converts analog voltage signals from PMTs to digital signals that are displayed graphically on flow data plots.
- Antibody cocktail** A combination of antibodies in a single tube to allow for staining of multiple samples or for purification of a cell population using columns or magnetic separation.
- Bandpass Filter** A filter that is positioned in front of a PMT that restricts the wavelengths of light allowed to reach that PMT.
- Biexponential scaling** Log-based scaling that allows one to see units of fluorescence below zero.
- Biotinylated antibodies** An antibody that has been modified by the addition of a biotin group.
- Blocking/Neutralizing antibody** An antibody that binds the active site of a protein that prevents the interaction of that protein with its ligand(s).
- Brefeldin A** A protein transport inhibitor that functions by blocking export of proteins via the Golgi apparatus. Rather, it restricts their localization to the ER.
- Brilliant Violet dyes** Dyes specific to the Violet laser that are extremely bright and photostable. Some exist as tandems to offer more options at a higher wavelength on the Violet laser.
- Camptothecin** A chemical compound that is a topoisomerase inhibitor derived from the *Camptotheca* tree. It binds to the DNA complex and prevents DNA re-ligation, causing DNA damage and apoptosis.

Cell enrichment The process of increasing the purity of a given cell population. This can be accomplished either by removing unwanted cells or selecting for a particular cell type.

Cell Fixation Chemically cross-linking proteins within a cell using a fixative agent (ex. Formaldehyde).

Cell Permeabilization Chemically disrupting the cell membrane by creating holes that allow antibodies access to intracellular proteins using plant-based (ex. Saponin), alcohol-based (ex. Methanol), or detergent-based buffers (ex. Triton X-100).

Cell proliferation dyes Amine-reactive dyes that covalently label the cytoplasm in live cells, and as the cells divide, the dye is diluted out. With each cell division, the daughter cells inherit roughly half the fluorescent label.

Cell purity The percentage of a desired cell population after cell enrichment.

Cell reactivation The process of stimulating either a differentiated or activated cell population to induce cytokine secretion.

Cellular Impedance The interruption of an electrical current by a cell between two electrodes.

Channel Also known as a photo multiplier tube (see PMT).

Chemokine receptors Seven transmembrane domain G-protein coupled receptors that bind chemokines.

Chemokine A protein that induces chemotaxis or trafficking/migration of a cell from one location to another.

Compensation Bead A polystyrene bead used during the process of compensation. Positive control compensation beads bind species-specific flow cytometry antibodies providing a positive fluorescence signal. Negative control compensation beads do not bind flow cytometry antibodies and provide a negative signal.

Compensation Matrix A matrix inversion of the spillover matrix values (i.e., $\text{SpilloverMatrix}^{-1}$).

Compensation Value The mathematical percentage of one fluorochrome that is subtracted away from another fluorochrome with which its spectrum overlaps.

Compensation The process of mathematically eliminating emission spectral overlap of one fluorochrome from another. This can 1) be an automatic feature of a given cell acquisition software (e.g., FACSDiva®) whereby the software mathematically calculates the compensation values for the experimenter, or 2) can be done manually by the researcher, often by manually adjusting the compensation values until the mean fluorescence intensities for two populations are equal.

Conjugated antibody An antibody that has been modified by the addition of a fluorochrome.

Conjugation The process of using chemistry to attach a fluorochrome to an antibody.

Costain Antibodies that are used in conjunction with the primary antibody of interest to define the phenotype and/or functional characteristics of a cell population.

Coulter Principle The principle that states in a fluid phase, the size of a cell may be determined by the volume of electrolyte it displaces as it passes through a pair of electrodes.

- CRISPR** Also known as CRISPR/Cas or CRISPR/Cas9. CRISPR stands for Clustered Regularly Interspaced Short Palindromic Repeats and is a prokaryotic immune response mechanism that confers protection against foreign DNA by recognizing and cutting DNA. When used to edit genomes, the Cas9 nuclease, guided by a synthetic RNA (gRNA), cuts the genome at a specific location allowing for genes to be inserted or removed.
- CyTOF** Cytometry by Time of Flight where mass spectrometry and flow cytometry are combined. Antibodies are labeled with heavy metal ion tags rather than fluorochromes.
- Cytokine** Secreted proteins that are released by cells that induce specific downstream interactions and communication between different cell types.
- Dead cell exclusion dyes** An amine reactive dye that preferentially labels intracellular proteins in dead cells based on the disrupted membrane integrity of dead cells versus live cells.
- Dead cell exclusion** The process of gating out dead cells using light scatter or dead cell exclusion dyes.
- Dendritic cells** An antigen presenting cell within the immune system capable of activating innate and adaptive immune cells.
- Density plots** Two-dimensional plots that can be used to examine cell size and granularity (FSC/SSC), protein fluorescence vs. SSC (or FSC), or protein 1 fluorescence vs. protein 2 fluorescence. Contours are used to show an accumulation of cells.
- Detector** Also known as a photo multiplier tube (see PMT).
- Dot plots** Two-dimensional plots that can be used to examine cell size and granularity (FSC/SSC), protein fluorescence vs. SSC (or FSC), or protein 1 fluorescence vs. protein 2 fluorescence.
- Doublet exclusion** Elimination of dead cells in the live cell gate using FSC-A vs. FSC-H. Dying cells aggregate and appear twice as big using FSC-A and FSC-H whereas singlets or live cells are in the diagonal that extends from the lower left corner to the upper right corner.
- Emission Maximum** The wavelength at which light emitted by a fluorochrome is at its maximum intensity.
- Emission Spectrum** The range of wavelengths of photons of light emitted by a fluorochrome when excited by a laser.
- Emission Wavelength** The range of the wavelengths of photons of light emitted by the electrons of an excited fluorochrome.
- Enzymatic dissociation** The process of using an enzyme, such as trypsin, to release adherent cells from a surface.
- Excitation Maximum** The wavelength at which a fluorochrome most optimally absorbs light energy from an excitation laser.
- Excitation Wavelength** The range of the wavelengths of photons of light that can be absorbed by a fluorochrome.
- FACS** Fluorescence Activated Cell Sorting. The method of enriching heterogeneous cell populations on a per cell basis using a flow cytometer with sorting

capability. Sorting is achieved using light scatter and fluorescence characteristics of cells stained with fluorochrome-conjugated antibodies.

Fc receptor A protein expressed on cells that specifically bind classes of immunoglobulin.

Flow cell The place within the cytometer where the cells are hydrodynamically focused into a single-cell stream.

Fluidic-based Flow Cytometry Flow Cytometry that uses the differential flow rates of the sheath and sample stream to align cells into a single file order for interrogation by the excitation lasers.

Fluorescence Spillover The detection of one fluorochrome by the PMT for a second fluorochrome as the result of spectral overlap between the emission spectrums of both fluorochromes.

Fluorescence The emission of light from fluorochromes on conjugated antibodies caused by excitation of lasers on the flow cytometer.

Fluorochrome (or fluorophore) A fluorescent dye conjugated to a flow cytometry antibody that absorbs laser light at one wavelength, is excited, and then emits its own light at a different wavelength.

Fluorokines Cytokines that are conjugated to fluorochromes and are used identify cells that express the receptor for that cytokine.

Fluorophore see Fluorochrome.

FMO *Fluorescence Minus One* is a type of flow cytometry control in which cells are labeled fluorochrome-conjugated antibodies in a panel except one.

FSC *Forward Scatter* is measured by light scattered forward and is used to measure cell size. FSC also takes into account nuclear to cytoplasmic ratio, membrane topography, and other cellular characteristics.

Gain An adjustment made to increase or lower the PMT voltage by regulating the sensitivity of the PMT. The voltage serves as the preamplifier gain and dictates how much PMTs amplify light signals by increases or decreases in voltage. Adjustments to gain will impact your detection threshold; increases will improve signal brightness but also increase the background.

Gating Selecting an area of a flow plot to focus on. Linear gates are used on histograms whereas polygons, rectangles, or circles can be used to gate on dot plots.

GFP *Green Fluorescent Protein*. Typically used in knock-in mouse strains and transfectants to label a cell population or marker of interest.

Granulocytes An innate white blood cell with secretory granules and tri-segmented nuclei (ex. eosinophil, basophil, neutrophil). Also known Polymorphonuclear leukocytes or PMN, PML, or PMNL.

High-density markers Proteins that are expressed at high levels on a particular cell population.

Histograms Flow plot that can be used to measure antibody fluorescence (x-axis) versus cell number (y-axis).

Hybridomas A chemical fusion between an antibody-producing B cell and immortalized cancerous myeloma cell that continuously makes unlimited amounts of antibody.

Hydrodynamic focusing This process uses differential fluid flow (cell sample stream versus sheath fluid stream) rates to align cells single file in the flow cell of a flow cytometer.

Immunogen An antigen, typically foreign, or substance that elicits an immune response.

Interrogation point The place within a flow cell of a flow cytometer where the cells interact with the excitation laser(s).

Ionomycin (Calcium Ionomycin) Calcium ionophore that stimulates intracellular cytokine production in a non-antigen-specific manner.

Isotype controls An antibody that lacks specificity to a target antigen but matches characteristics of the antigen-specific primary antibody.

iTregs Induced T regulatory cells that are characterized by the expression of FoxP3, CD4, and CD25 (among others) and are induced by TGF β and IL-2.

Jablonski Diagram An illustration created by Alexander Jablonski in 1933 depicting how resting electrons absorb energy, become excited, and then release energy in the form of photons of light as they return to a resting state.

K562 An immortalized myelogenous leukemia cell established from a Chronic myelogenous leukemia (CML) patient commonly used as targets for NK cells.

Knockout A cell or animal that has been genetically modified to be deficient for a gene or protein.

Lineage marker Proteins that are uniquely expressed by a given cell type and thus define that cell.

Low-density markers Proteins that are expressed at low levels on a particular cell population.

MFI Mean Fluorescence Intensity is used to describe the signal/fluorescence intensity of a fluorochrome-conjugated antibody. It is calculated using the geometric mean, mean, or median index.

Monensin A protein transport inhibitor that prevents protein secretion by interacting with the Golgi transmembrane Na⁺/H⁺ transport system.

Monoclonal Antibody An antibody that has monovalent affinity that binds a single epitope of the same target antigen.

Monocytes A phagocytic white blood cell that becomes a macrophage upon migration into tissues.

Negative selection Enriching a desired cell population by depleting unwanted cells using a cocktail of antibodies specific to those unwanted cells.

NK cell Degranulation The process by which an NK cell releases cytolytic granules upon activation.

NK cells A Natural Kill cell is a type of innate lymphocyte known for its ability to kill tumor cells and virally infected cells.

PBMCs Peripheral Blood Mononuclear Cells is any peripheral blood cell that has a round nucleus (includes T, B, NK, dendritic cells, and monocytes).

Photobleaching Fluorochromes that fade, degrade, or fall apart when exposed to light.

Photodiode A detector on a flow cytometer that converts light into an electrical current. Photodiodes differ from PMTs in that they are less sensitive to light

photons than a PMT and do not amplify the signal to the degree of a PMT. They are used to detect FSC.

Photostable Fluorochromes remain intact when exposed to light and are not prone to photobleaching.

PMA Phorbol 12-Myristate 13-Acetate chemical compound that is a Protein Kinase C (PKC)-activating phorbol ester that induces Calcium flux and nonspecifically induces cytokine production.

PMTs Photomultiplier tubes digitally convert emission spectra into data points into graphical form on the computer.

Polyclonal antibody A group of antibodies that bind to multiple epitopes of the same target antigen produced by multiple B cells.

Positive selection Enrichment of a cell population using single high affinity antibody specific for that population.

Pre- and post-sort The percentage of a desired cell population prior to (pre) or following (post) cell enrichment.

Primary antibody An antigen-specific immunoglobulin (antibody) that is not labeled with a fluorochrome.

Quadrant Each of four parts of a two-color dot plot which has been divided by two lines separating the positive and negative fluorescence populations for each color.

Reference beads A solution of beads at a known concentration, which when added to a single cell suspension can be used to calculate the concentration of the cells in the suspension.

Secondary Antibody A conjugated antibody that has specificity for another antibody. Typically, a secondary antibody is specific for all isotypes of a class of immunoglobulin from a given species (mouse, rat, hamster, etc.). Conjugated secondary antibodies are necessary for the detection of unconjugated antibodies by flow cytometers.

Spectral overlap Emission spillover from one fluorochrome into another. This occurs with fluorochromes that are close in wavelength to each other.

Spectrum Viewers Generally an online resource that displays the spectral overlap of fluorochromes graphically as histograms.

Spillover matrix table A table listing the spillover value for every combination of fluorochrome in a staining panel.

Spillover Matrix A table listing the fluorescence spillover values between all fluorochromes in a flow cytometry staining panel.

Splenocytes Immune cells found in the spleen.

SSC Side Scatter is measured by light scattered perpendicular to the laser beam and is used to measure cell complexity and granularity.

Staining volume The amount of flow cytometry staining buffer in which cells are resuspended when staining with fluorochrome-conjugated antibodies.

Staining The process of incubating cells with antibody/s to detect protein expression.

Stokes Shift The numerical difference, in nanometers, between the Excitation Maximum wavelength and Emission Maximum wavelength for a fluorochrome or fluorescent protein. Described by Sir George Stokes in 1852.

Strain-specific antibodies Antibodies that will specifically recognize proteins from one strain of mouse but not from other strains of mice.

Streptavidin A bacterial-derived protein with a high affinity for biotin. When conjugated to a fluorochrome, Streptavidin is used as a secondary reagent to detect biotinylated-primary antibodies.

T cells A cell of the adaptive immune system that develops in the thymus and is activated in an antigen-specific manner via their T Cell Receptor (TCR).

Tandem dyes Covalent coupling of two fluorochromes where one serves as an acceptor molecule and the other serves as a donor to create a new fluorochrome. Tandem dyes have the excitation properties of the donor and emission properties of the acceptor (ex. PE-Cy7; PE is the donor and Cy7 is the acceptor molecule).

Threshold A setting used to digitally filter out any artifacts that are not cellular events during acquisition based on minimum size. Events below the threshold are not collected during acquisition.

Transfectant A cell that has been genetically modified to express a non-native protein by the process of transfection.

Tregs Naturally occurring T regulatory cells that are characterized by the expression of FoxP3, CD4, and CD25 (among others).

Unconjugated antibody A native antibody lacking a fluorochrome for detection.

Validation The process by which an antibody is tested and characterized to ensure that it binds to a specific antigen and does not possess nonspecific binding properties.

Voltage Also known as the pre-amplifier gain. The voltage setting dictates how much the detector, or photo multiplier tube, amplifies the light signal from a fluorochrome by applying a higher or lower voltage to that detector.

Index

A

Abort rates, 14, 15
Acoustic-based flow cytometry, 9
Activation markers, 94, 96
Alcohol-based permeabilization buffers, 136
Alexa Fluors, 41, 42
Allophycocyanin (APC), 8, 54–58, 60, 61, 63–71
Analog to digital converters (ADCs), 20
Antibody development
 antigen design, 75
 applications, 76
 cell models, 76
 immunogens, 75
 Ki-67/MKI67, 78
 R&D systems, 76
 transfectants and natural cell subsets, 77
 validation, 75, 77
 Western blot, 75
Arithmetic mean, 28
AutoCompensation, 60, 61, 63, 66, 68, 69

B

Bandpass filters, 37, 38, 54, 56
BD CellQuest™, 66
BD FACSCalibur™, 20, 46, 66
BD FACScan™, 17
BD FACSDIVA™, 20, 28
BD LSRFortessa™, 46, 47, 71
Biexponential vs. logarithmic scaling, 28–30
Biological cell models, 2
Biotinylated antibody, 150, 151, 153
 staining order, 194
Bovine serum albumin (BSA), 157

Brefeldin A, 137
Brilliant Violet dyes, 41

C

C57Bl/6 mice, 188, 189
CD3+ T cells, 186
CD4 expression, 198
CD11c expression, 199
CD11c on human M2a macrophages, 200
CD19 biotinylated antibody, 193
Cell activation controls
 artificial adoptive transfer systems, 95–96
 CCR6, 95
 CD45RO, 95
 CD69, 92
 CD127 expression, 95
 FMO control, 93
 IL-7 receptor, 94
 in vitro stimulation, 95
 naive cells, 93
 T cells, 95
 unstimulated cells, 93
Cell activation reagents, 147
Cell enrichment, 150
 columns, populations, 149
 FACS, 153
 general principle, 149
 hCD4+ Memory T cell purification, 152
 magnetic purification, 150
 magnetic selection (*see* Magnetic cell selection)
 negative selection, 149
 sorting, BD FACSAria™, 154
 T cell subset columns, 149

- Cell proliferation dyes
 - antibodies, 123
 - CD3⁺ T cells, 123
 - CD25⁺ T cell population, 123
 - detect BrdU/Ki-67, 121
 - fluorochromes, 123
 - individual cell division, 123
 - Cell purification kits, 147, 155
 - Cell purity, 150, 151
 - Cell surface staining
 - secondary antibodies, 187
 - Cellular impedance, 4, 5
 - Cell viability dyes, 108
 - Chemokine receptors, 126, 127, 158, 166
 - Circle gate, 27
 - Cluster of Differentiation (CD), 76
 - Compensation, 66
 - A647 PMT, 37
 - APC and A700, 55–58
 - AutoCompensation software, 60
 - bandpass filters, 37, 56
 - beads, 34, 37, 58–61, 64–66, 73
 - CD3 FITC and CD19 APC antibodies, 55
 - CD4⁺ T cells, 54, 64
 - CD19⁺ B cells, 54
 - cell populations, 58
 - conjugated antibodies, 58
 - cytometer, 60
 - definition, 36–37
 - experiments, 67, 69, 70
 - FACSDiva®, 37
 - FITC, 54
 - fluorescence spillover, 36
 - fluorescence staining patterns, 60, 61
 - fluorochromes, 37, 56, 58, 70–72
 - human lymphocytes, 54
 - lights, 64
 - lymphocytes, 55
 - manual, 66
 - materials, 73–74
 - matrix, 64
 - MFI (*see* Median fluorescence intensity (MFI))
 - NK cells and B cells, 55
 - PMT, 63
 - single stain controls *vs.* beads, 73
 - spectral overlap, 37, 38, 53, 54
 - spectrum viewers, 38
 - spillover values, 61–63
 - wavelengths, 64
 - Costain, 160–163, 165, 166, 170–172, 175, 177, 180, 187, 188
 - Coulter principle, 4, 5
 - CRISPR knockout, 79
 - Cytometer setup and tracking (CS&T), 33
- D**
- Data plots, 25–27
 - Data sets, 103, 108, 120, 135
 - antibody, concentration, 116, 117
 - block, Fc receptors (*see* Fc receptor block)
 - CD127 antibody, 116
 - cell number, 114, 115
 - exclusion, dead cell (*see* Dead cell exclusion)
 - intracellular staining (*see* Intracellular staining)
 - surface staining (*see* Surface staining)
 - titrating flow cytometry antibodies, 117, 119
 - Dead cell exclusion
 - amine-reactive viability dye, 109
 - cell size, 112, 113
 - detection, early and late apoptotic events, 110, 111
 - dyes and proteins, 108
 - "false positive" staining, 113
 - fixable viability dyes, 109
 - flow cytometry antibodies, 108
 - fluorescence, aqua viability dye, 111, 112
 - fluorescently labeled Annexin-V protein, 109
 - IFN γ and LPS polarized macrophages, 111, 112
 - method, detecting apoptosis, 109, 110
 - nucleic acids, 108
 - Density plots, 21, 22, 25–27
 - Diluting perm buffers, 184
 - Directly conjugated antibody, 158, 161–164, 166, 167, 170, 172, 175–178, 180
 - Dot plots, 21–23
 - Doublet exclusion, 31, 32
 - DyLight dyes, 41
- E**
- Electronics system
 - ADCs, 20
 - BD FACSCalibur™, 20
 - density plots, 21, 22
 - dot plots, 21, 22
 - emission spectra into digital signals, 20
 - flow data plots, 21
 - histograms, 22
 - ELISA, 2
 - Emission maximum, 19, 20
 - Emission spectrum, 54, 56, 58, 63, 70, 71
 - Excitation maximum, 19, 20
 - Expression of tagged proteins, 77

F

- FACSDIVA™, 23, 60, 63, 66
- Fc receptor block, 162, 163
- antibodies/immunoglobulin, 103
- basic structure, antibody, 104
- common, human, 103, 105
- human IgG, 191
- human monocytes, 104, 190
- Ig gamma class, 190
- immune cell types, 103
- interaction, 104
- isotype control staining, 106
- monomeric IgG vs. IgG (immune)
- complexes, 106
- mouse anti-human SLAM F7 antibody, 104
- mouse IgG2a isotype, 104
- Ms IgG2a antibody, macrophages, 107
- SLAM F7, macrophages, 107, 108
- Fetal bovine serum (FBS), 157
- Fetal calf serum (FCS), 157
- FITC/A488-conjugated antibody, 189
- Fixation, 168, 174–180
- Flow antibodies, 145, 146, 154
- Flow cytometry, 13, 75, 78, 157–159, 164–166, 168
- acoustic-based, 9
- antibodies, 2 (*see also* Antibody development)
- antigen-specific vs. isotype control antibodies, 87
- biomedical and clinical research, 1
- biotinylated antibodies
- biotin-streptavidin interaction, 164
- in PBMCs, 165–166
- in WB, 164–165
- cell activation controls, 92
- cell populations, 3, 4
- cellular impedance, 4, 5
- chemokine receptors, 166–167
- components, 1
- Coulter principle, 4, 5
- CyTOF, 9–11
- definition, 2–4
- FACS, 7
- fluidics-based, 8 (*see also* Fluidics-based flow cytometry)
- fluorescence-based, 5
- fluorescent dyes, 2
- fluorescent molecules, 7
- FMO, 96
- instrument, 1, 2
- internal lineage controls, 88
- intracellular staining, 168–181
- fixation and permeabilization, 168
- formaldehyde/methanol staining (*see* Formaldehyde/methanol staining)
- formaldehyde/saponin IC staining (*see* Formaldehyde/saponin IC staining)
- FoxP3/transcription factor buffer (*see* FoxP3/transcription factor buffer)
- isotype controls, 84
- LAMP proteins, 167–168
- monoclonal antibody, 6
- mouse immunoglobulin isotypes, 85
- patient testing, 1
- phenotype live cells, 1
- polyclonal antibody, 6
- primary (*see* Primary antibodies)
- protein expression, 1
- staining buffers
- Ca²⁺ and Mg²⁺ ions, 158
- cell aggregation, 158
- CO₂ concentrations, 157
- DNase and EDTA, 158
- FACS buffer, 157, 158
- HBSS contains glucose, 157
- proteins, 157
- surface and intracellular, 158–159
- surface staining, 160–164
- LAMP proteins, 159
- PBMCs (*see* Peripheral blood mononuclear cells (PBMCs))
- whole blood (*see* Whole blood (WB))
- visualizing and identifying, 1
- Flow cytometry staining buffer (FACS), 157, 158
- FlowJo®, 28, 34
- Fluidics-based flow cytometry, 1, 8, 36
- bandpass filters, 23
- compensation (*see* Compensation)
- CS&T, 33
- dot plots, 22, 23
- electronics system, 20–22
- fluidics system, 13–15
- Fortessa, 34, 35
- FSC, 22
- iTregs, 33, 35, 36
- materials, 23–24, 39–40
- optics system, 15–20
- panel design, 33
- sample acquisition and analysis, 34, 36, 37
- SSC, 22
- Fluidics system
- abort rates, 14, 15
- digitally filter, 14
- hydrodynamic focusing, 13, 14
- laminar flow, 13, 14
- sample stream vs. sheath fluid stream, 13
- single stream, 13

- Fluorescein isothiocyanate (FITC), 7, 8, 18, 54–57, 60, 61, 63–65, 71
 Fluorescence, 27
 antibody, 7, 25
 MFI (*see* Mean fluorescence intensity (MFI))
 Fluorescence activated cell sorting (FACS), 7
 flow cytometry, 153
 separation, live cell populations, 153
 T-cells (CD3⁺) and B-cells (B220⁺), 153
 Fluorescence amplification, 81
 Fluorescence-based flow cytometry, 5, 6
 Fluorescence Minus One (FMO)
 background surface staining, 99
 defined, 96
 Fc receptors, 99, 100
 fluorescence intensity, 100
 fluorochrome, 99
 GRO α PE, 97
 human monocytes, 97, 99
 IL-6 expression, 97
 vs. isotype controls, 97
 LPS-stimulated cells, 100
 MFI, 100
 mouse and human cells, 98
 mouse splenocytes, 96, 97
 Fluorescence spillover, 53
 APC, 60, 64, 71
 compensation (*see* Compensation)
 and spectral overlap, 53
 values, 55
 Fluorescent dyes, 2
 Fluorescent molecules, 7
 Fluorochrome-coupled antibodies, 2
 Fluorochromes, 1, 2, 7–9, 13, 16–22, 26–28, 34, 36–38, 78, 159, 160, 164–166
 Alexa Fluors, 41, 42
 and antigen density, 53
 bandpass filters, 56
 BD FACSCalibur™ (Calibur), 46
 BD LSRFortessa™ (Fortessa), 46, 47
 definition, 58
 detector, 56
 dimming, 70
 DyLight dyes, 41
 emission spectrums, 56, 58, 63
 FITC, 42
 fluorochrome-conjugated antibodies, 58
 Fortessa *vs.* Calibur, 47, 48
 high-density markers, 42
 high spectral overlap, cell types, 71, 72
 Janelia Fluor® dyes, 41
 low-density markers, 43
 Novus Biologicals, 41, 42
 overlapping, 53, 70
 PE and APC, 42
 photobleaching, 42
 photostability, 41
 PMTs, 54
 signal intensity, 43
 spectral overlap, 43, 46
 spectrum viewers, 41, 43, 44, 46
 spillover matrix, 61
 Stokes shift, 41
 tandem dyes, 42
 UV and Violet laser, 42, 43
 wavelength, 41, 63
 Fluorokines, protein detection, 132
 Formaldehyde, 135, 136, 139, 141, 143, 145, 168–174, 178–181
 Formaldehyde/methanol staining, 179–181
 PBMCs
 conjugated phospho-proteins, 180–181
 unconjugated phospho-proteins, 179–180
 phospho-proteins, 178
 Formaldehyde/saponin IC staining, 168–174
 PBMCs
 conjugated antibodies, 173–174
 unconjugated antibodies, 171–173
 WB
 conjugated antibodies, 170–171
 unconjugated antibodies, 168–170
 Fortessa, 34, 35
 Forward scatter channel (FSC), 15–17, 19–22, 25, 27, 30–32, 34
 FoxP3, 135, 140–142, 144
 diluent, 174–176, 178
 fixation buffer, 175, 176, 178
 permeabilization buffer, 175, 177, 178
 FoxP3/transcription factor buffer, 174–178
 PBMCs
 conjugated antibodies, 177–178
 unconjugated antibodies, 176–177
 WB
 conjugated antibodies, 175–176
 unconjugated antibodies, 174–175
 Fragile cells, 158

G
 Gating, 26–28, 31, 34, 36
 Genetic strategy, 77
 Geometric mean MFI, 28, 29

H

Hanks balanced salt solution (HBSS), 157,
158, 168–171, 174, 175, 179, 180
hCD4+ Memory T cell purification, 152
Hierarchy, 27, 34
High-density markers, 42, 43, 47–50
Histograms, 25–28, 38
HLDA10 validation process, 78
Human CD3 HEK eGFP transfectants, 189
Human Cell Differentiation Molecules
(HCDM), 76
Human IgG block, 160–165, 167, 168, 170,
171, 173–177, 179, 180
Human Leukocyte Differentiation Antigen
(HLDA), 77
Human lymphocytes, 54
Human lyse buffer, 160–162, 164, 165,
168–170, 175, 176, 180
Human peripheral blood cells, 2, 3
Hybridomas, 6
Hydrodynamic focusing, 13–15

I

IL8 in monocytes, 192
Immunocapture mass spectrometry, 77
Immunocytochemistry (ICC), 2
Immunofluorescence (IF), 4
Immunohistochemistry (IHC), 2, 4
Independent antibody strategy, 77
Internal lineage controls
bimodal distribution, 88
cell populations, 88
distinct populations, 91
Fc receptors, 92
granulocytes, 88
lymphocytes, 88, 90
macrophages, 91
monocytes, 90, 91
NK cells, 89
positive and negative gates, 89, 90
T cell development, 89, 90
International Working Group for Antibody
Validation, 77
Interrogation point, 153
Intracellular markers, 168
Intracellular staining
buffer systems, 135, 136
fixation/staining order, 145
formaldehyde, 143
FoxP3, 142

methanol-sensitive fluorochromes, 145
optimizing fixation, 139
order, 140, 141
paraformaldehyde, 144
phosphorylated proteins, 142
phospho-Stat 1, 145
secreted cytokines, 137, 138

In vitro-activated T cells, 92

Isotype controls, 146, 154
antigens, 86
APC voltages, 87
bi-modal populations, 88
characteristics, 88
fluorochrome, 86
human CD4 protein, 85
IgG2a, 86
KLH antigen, 85
lymphocytes, 86
mice and humans, 84
PMT voltages, 86
primary antibody, 86
iTregs, 33–36

J

Jablonski diagram, 18, 19
Janelia Fluor® dyes, 41

K

Killing assays, 123, 129

L

Laminar flow, 13, 14
LAMP proteins, 128, 131, 159, 167–168
Lasers
BD LSR Fortessa™, 19
beams to scatter light, 15
red, 17, 42
single stream, 13
SSC, 15
strength, 47, 50
ultraviolet, 17, 42
violet, 17, 42
wavelengths, 17
yellow-green, 17
Logarithmic vs. biexponential scaling, 28–30
Lonomycin, 137–139, 141
Low-density markers, 43, 48–50
Lymphocytes, 31

M

MagCelect™, 155
 Magnetic cell selection
 applications, 150
 biotin-conjugated antibody, 150
 nanoparticles, 150
 negative, 151, 152
 positive, 152
 purifications, 153
 recovery, 153
 splenocytes, 150
 streptavidin ferrofluid, 150
 Manual compensation, 63, 66, 69
 Mass cytometers (CyTOF), 9–11
 Mean fluorescence intensity (MFI), 28, 29
 Mean MFI, 28
 Median fluorescence intensity (MFI), 28, 29,
 47, 48
 cell populations, 66, 67
 Methanol, 135, 136, 143, 145
 permeabilization, APC conjugated
 markers, 184, 185
 resistant fluorochromes, 159
 sensitive fluorochromes, 159
 treatment, PE fluorescence, 185
 Mice, 85
 Mixed cell populations, 2
 Monensin, 129, 137–139, 141, 147
 Monoclonal antibody, 1, 6–9
 Monocytes, 31, 91
 Mouse IgG block, 160–165, 167, 168,
 170–173, 175–181, 188
 Mouse lyse buffer, 159, 161

N

Naïve vs. blasting T cells, 31
 Natural killer (NK) cells, 160, 162, 165, 167
 Negative selection, cell enrichment, 149–151
 NK cell degranulation, 128–131
 NK cell killing assay, 198
 NK1.1 expression, 188, 189

O

Optics system, 15
 amplifier controls, 20
 bandpass filters, 20
 BD FACScan™, 17
 detection, antibody-labeled cells, 17, 18
 detectors/channels, 15
 and electronics, 16
 emission maximum, 19, 20
 excitation maximum, 19, 20
 FSC, 15–17

Jablonski diagram, 18, 19
 laser beams (*see* Lasers)
 photodiodes, 15
 pianist, 20
 PMTs (*see* Photomultiplier tubes (PMTs))
 SSC, 15–17
 Stokes shift, 19, 20
 wavelengths, 19
 Orthogonal strategy, 77

P

Parafilm®, 179, 180
 Peridinin-chlorophyll Protein Complex
 (PerCP), 8
 Peripheral blood mononuclear cells (PBMCs)
 biotinylated antibodies, 165–166
 conjugated antibodies, 163–164
 formaldehyde
 methanol staining, 179–180
 saponin IC staining, 171–174
 FoxP3/transcription factor buffer staining,
 176–178
 unconjugated antibodies, 162–163
 Permeabilization, 136, 137, 159, 168, 175,
 183, 184
 Phosphate buffered saline (PBS), 157, 158,
 168–180
 Phospho-proteins, 178, 180–181
 Photobleaching, 41, 42
 Photodiodes, 15
 Photomultiplier detection, 8
 Photomultiplier tubes (PMTs), 15–20, 22, 23,
 54, 56–58, 60, 63–65, 68, 70–72
 Photostable, 41
 Phycoerythrin (PE), 8
 Polyclonal antibody, 6
 Polygon gate, 27, 31, 34
 Positive selection, cell enrichment, 150, 153
 Primary antibodies, unconjugated, 78–83, 160,
 163, 164, 169, 172, 192
 CD19, 81, 83
 donkey anti-mouse IgG antibody, 80
 fluorochrome, 78, 79
 goat anti-mouse IgG antibody, 80
 human CD3, 81, 83
 immune system, 80
 immunoglobulin, 79
 monoclonal antibody, 80
 NK cells, 82
 staining process, 82
 Proliferation and viability dyes and reagents,
 147
 Protein expression, 1
 Pseudocolor dots plots, 21–22

Q

Quadrants, 26, 27, 34
Quality control, 77

R

R&D systems flow antibody validation
 process, 78
Reactivation with PMA, 191
Reagents, 7
Rectangle gate, 27
Red blood cells (RBCs), 161, 162
Reference beads, 198

S

Sample fluid stream, 13
Saponin, 135, 136, 139, 142, 168–174
Secondary antibody, 160, 161, 163, 164, 169,
 172, 175, 177, 180, 187, 188, 192
 CD19 and CD3, 82
 flow cytometry, 80
 goat anti-mouse IgG, 80, 81
 isotype, 79
 PE fluorescence, 83
 polyclonal antibodies, 80
 species, 79, 80
Sheath fluid stream, 13
Side scatter channel (SSC), 15–17, 19–22,
 25–27, 30, 31, 34
Signal intensity, 43, 48
Single stain controls vs. beads, 73
Sodium azide, 158, 166, 167
Spectral overlap, 37, 38, 43, 46, 47, 50, 53–56,
 61, 64, 65, 71–73
Spectrum viewers, 37, 38, 41, 43, 44, 46, 50,
 56
Spillover matrix table, 63
Spillover values, 55, 56, 61, 63, 64, 66–70, 73
Staining buffers/Fc block reagents, 148, 155
Stokes shift, 19, 20, 41
Strain-specific antibodies, 188
Streptavidin ferrofluid, 150, 153
Surface staining
 antibody concentration, 194, 195

CD3, CD19 and CD14, 133
chemokine receptors, 126, 127
detection, NK cells, 128–131
HLA-DR expression, 133
myeloid dendritic cells, 134
parameters, flow cytometry, 119, 120
plasmacytoid dendritic cells, 134
protein detection, fluorokines, 132
reagents, assess cell proliferation, 121–123
tracking cell populations, mice, 125
traditional lineage markers, 134
volume, 195

T

Tandem dyes, 41, 42
T cells, 191
Transfectants, 190
Transfected cell lines, 2
Tregs, 118, 120, 142

U

UltraViolet dyes, 41
Unconjugated antibody, 159–164, 168–177
UV laser, 8
UV/Violet fluorochromes, 120

V

Violet laser, 8
Voltages, 34

W

Wavelength, 41, 46
Western blot (WB), 2–4, 75
Whole blood (WB)
 biotinylated antibodies, 164–165
 conjugated antibodies, 161–162
 formaldehyde/saponin IC staining,
 170–171
 FoxP3/transcription factor buffer staining,
 175–176
 unconjugated antibodies, 160–161